

THE ISOLATION CULTURE AND CHARACTERISATION OF GRAM-
NEGATIVE NON-SPORING ANAEROBIC BACILLI WITH SPECIAL
REFERENCE TO THE OCCURRENCE OF BACTEROIDES SPECIES
IN THE HUMAN MOUTH

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FRONTISPIECE



Colonial appearance of Bacteroides
melaninogenicus NCTC 9338 on
lysed-blood agar

PREFACE

A survey of the literature clearly indicates that the state of the classification of the Gram-negative anaerobic bacilli has been confused. Although some clarification has been made several problems still exist. Many of the identification schemes available involve complex procedures that are not directly applicable to the clinical bacteriology laboratory. Anaerobic culture techniques have improved and it has been shown in this laboratory that careful use of relatively simple, conventional, anaerobic techniques can give satisfactory results with many test systems (Watt, 1972a and b). A result of the technological and manipulative advances has been an increasing awareness of the presence of Bacteroidaceae in the commensal flora of man and animals and of the potentially pathogenic role of some members of this family.

Thus the purpose of the present study was to develop for the identification of Gram-negative, non-sporing, anaerobic bacilli a scheme which would be of use in the clinical laboratory and which, if possible, applied suitably poised conventional laboratory tests to the characterisation of these organisms. Strains isolated from clinical material were used to evaluate this identification scheme. It became apparent that some improvements in sampling techniques were required, especially for the isolation of organisms from the mouth. Accordingly a number of media, including selective and transport media, were studied in the laboratory and the most useful of the media tested were selected for use in a clinical sampling

survey in which the various media could be evaluated. The survey involved the isolation of oral, Gram-negative anaerobes from the gingival crevice. As well as providing a clinical evaluation of the various isolation methods and media, the organisms isolated were characterised by the identification scheme. This has provided information on the range of Gram-negative, non-sporing anaerobes resident in the normal oral flora and might usefully provide a basis for the further elucidation of the role of these commensals in the mouth.

ABSTRACT OF THESIS

A scheme for the identification of Gram-negative, non-sporing, anaerobic bacilli is presented. This is based on the results obtained with strains obtained from culture collections, strains referred by other interested workers, isolates from clinical specimens, isolates from normal human faeces, and isolates from subgingival dental plaque.

The tests employed were conventional bacteriological tests adapted for use with these organisms. They included: tests of tolerance to certain dyes and bile salts; resistance patterns obtained with antibiotic disks; and a number of biochemical tests including carbohydrate fermentation tests.

A clear distinction could be made between the various reference strains - Bacteroides fragilis, B. melaninogenicus, B. oralis, Fusobacterium necrophorum and F. polymorphum being the main species recognised. Most B. fragilis and B. melaninogenicus strains could be placed into one of the recognised subspecies, but some organisms gave patterns of results intermediate between those of recognised subspecies. Within the particular species, strains of B. fragilis and B. melaninogenicus seem to form a continuum of variants with clusters of strains in the designated subspecies.

A number of strains of B. melaninogenicus were isolated that would only grow in mixed culture. In efforts to solve this problem several improved media were tested in the laboratory and

some were evaluated in the clinical situation. The transport of specimens in Möller's VMG II transport medium allowed recovery of B. melaninogenicus strains comparable with that obtained by the direct plating of the sample. Incorporation of kanamycin and vancomycin into the plating medium enhanced recovery of B. melaninogenicus and permitted growth of the other recognised species of oral, Gram-negative, anaerobic bacilli.

The strains of B. melaninogenicus ss. melaninogenicus and B. oralis isolated in this survey were characterised by the tests developed in the first part of the study. The taxonomic significance of the results is discussed.

ACKNOWLEDGEMENTS

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The technical help of Mr. R. Brown and his staff in the Microbial Pathogenicity Research Laboratory of the Department of Bacteriology, University of Edinburgh, has been much appreciated and I should like to record my thanks.

The development of concepts in this field with colleagues in the Microbial Pathogenicity Research Laboratory has been a stimulating experience and I am grateful to them.

I should like to express my thanks to the many clinical dental students, other students, colleagues and staff of the Departments of Bacteriology and Dental Surgery who so willingly allowed samples to be collected for the isolation of Gram-negative anaerobes. The co-operation of the nursing staff of the Dental Hospital was much appreciated. I am also most grateful to the many colleagues who referred strains for this study.

In the preparation of this thesis my grateful thanks are due to the staff of the Central Medical Library of the University of Edinburgh for their help and forbearance especially with

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Throughout this work the financial assistance of the University of Edinburgh Faculty of Medicine Scholarship Fund has been much appreciated.

The results of some of the work presented in this thesis have already been reported. The relevant references are:

(i) HOLBROOK, W.P. and DUERDEN, B.I. (1974).

A comparison of some characteristics of reference strains of Bacteroides oralis with Bacteroides melaninogenicus. Archs. oral Biol., 19, 1231-1235.

(ii) DUERDEN, B.I., HOLBROOK, W.P., COLLEE, J.G. and WATT, B. (1976). The characterisation of clinically important Gram-negative anaerobic bacilli by conventional bacteriological tests. J. appl. Bact., 40, 163-188.

(iii) DUERDEN, B.I., HOLBROOK, W.P., COLLEE, J.G. and WATT, B. (1976). A scheme for the identification of clinically important Gram-negative anaerobic bacilli by simple bacteriological tests. Proc. Path Soc. Great Britain and Ireland: J. med. Microbiol., 9, Px, Abstract no. 49.

(iv) HOLBROOK, W.P. (1976). The isolation of Bacteroides melaninogenicus from the human mouth. Int. Ass. Dent. Res., British Division, Abstract no. 81, (J. dent. Res., in press).

The investigations and procedures that form the basis of this thesis were personally designed and performed or supervised by the author. It has been a privilege to be involved in the characterisation studies with colleagues in the Microbial Pathogenicity Research Laboratory and to be associated with them in the development of several concepts in this field.

H.P. Holbrook

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SUMMARY

The literature on the evolving classification of Gram-negative, non-sporing anaerobic bacilli of medical and dental interest is reviewed. Particular reference has been made to their commensal role in man and animals and to their role in disease. Gram-negative, non-sporing anaerobes have been implicated, usually in mixed culture, in a wide variety of infections in animals and humans. The occurrence of these organisms in the human mouth and their involvement in inflammation of the dental pulp, periodontal disease and other infections in the human mouth is specially considered.

Methods of identifying Gram-negative, non-sporing anaerobes are described. These are based on results with: (i) tests of tolerance to bile salts and dyes; (ii) antibiotic-disk-resistance tests; and (iii) biochemical tests including carbohydrate fermentation tests. A scheme for the identification of these organisms has been developed and is presented. An evaluation of the scheme has been made with a wide range of isolates from clinical material and from samples collected from healthy human mouths.

Problems of growth were encountered with a number of strains and this led to the examination of several improved media. A liquid medium, solid medium, transport medium, enrichment medium and two selective media were developed. These were tested in the clinic. The results of the evaluation of these various media and

procedures for the isolation of B. melaninogenicus from the gingival crevice are presented. Möller's VMG II transport medium was found to be useful in maintaining the viability of these organisms in transit, that is prior to the inoculation of solid media. The enrichment broth developed gave no improvement in recovery of B. melaninogenicus over samples held in transport medium or plated directly at the chairside on to solid media.

A selective medium containing kanamycin and vancomycin was found to give a significantly better recovery of B. melaninogenicus than non-selective media. A sampling procedure derived from these studies was found to be reliable and it allowed all the known oral commensal species and subspecies of Gram-negative, non-sporing, anaerobic bacilli to be isolated. Other methods of isolation and culture are reviewed and discussed.

Although most of the strains isolated were successfully identified by the identification scheme, some other strains could not be allocated to any of the currently recognised species. These strains resembled B. melaninogenicus ss. melaninogenicus except that they failed to produce black or brown pigment. The taxonomic significance of these strains is discussed.

The degree of gingival inflammation had no effect on the observed success of any method of isolating B. melaninogenicus; which was used as an index of sampling efficiency. This indicates that the chosen technique of sampling is effective even in healthy subjects with lower numbers of organisms in the gingival crevice.

INTRODUCTION

The Gram-negative, non-sporing, anaerobic bacilli belong to the family Bacteroidaceae (Pribram, 1933). They are mostly pleomorphic, although there are some species with characteristic and uniform morphology, and most species are non-motile. Motile species possess peritrichous flagella. These organisms have been isolated from the alimentary tracts of man and other animals including cattle, poultry and some insects. They are also found in the mouths of man, some animals and in the genital tract of the human female (Holdeman and Moore, 1974).

Classification

Three genera are currently recognised in the 8th edition of Bergey's Manual (Holdeman and Moore, 1974): Bacteroides, Fusobacterium and Leptotrichia. Of uncertain affiliation, the genera Desulfovibrio, Butyrivibrio, Succinivibrio, Succinimonas, Lachnospira and Selenomonas are also noted. An alternative classification using, among others, the generic names Ristella, Zuberella, Eggerthella and Dialister has been used by a number of French bacteriologists (Prévot, 1938; Sebald, 1962; Beerens, Castel and Fievez, 1962) but these terms were not adopted for use by the International Committee on Nomenclature of Bacteria (Beerens, 1970). In that report the Taxonomic Sub-Committee for Gram-negative Anaerobic Rods recommended that the organisms variously classified as Ristella, Eggerthella or Bacteroides be collectively termed Bacteroides and that organisms assigned to the genera Fusobacterium and Sphaerophorus be placed in one genus, Fusobacterium. Organisms of the species previously classified as Fusobacterium fusiforme or F. plauti-vincentii have been found to differ biochemically from

other Fusobacterium species and have therefore been assigned to the genus Leptotrichia (Judicial Commission, 1954, Opinion 13).

The history of the classification of Gram-negative, non-sporeforming anaerobic bacilli is confusing. In the past, many organisms now classified as members of the family Bacteroidaceae have been identified as belonging to a wide range of other genera including: Fusiformis, Sphaerophorus, Pseudobacterium, Bacterium, Bacillus, Pasteurella, Ruminobacter, Fusocillus, Coccobacillus, Haemophilus, Actinomyces, Proactinomyces, Necrobacterium, Capsularis, Leptothrix, Vibrio, Streptothrix, Corynebacterium and Spirillum. The names used in this study correspond largely to those adopted by Holdeman and Moore in Bergey's Manual (1974) and proposed by Holdeman and Moore (1973) and Moore and Holdeman (1973).

Bacteroides

The genus Bacteroides was described by Castellani and Chalmers (1919) who referred to these organisms as anaerobic non-sporeforming rods but did not state any reaction to Gram-stain. Gram-positive organisms were excluded from the genus by Weiss and Rettger (1937). Veillon and Zuber (1898) studied the role of strictly anaerobic organisms in disease and named one of their isolates Bacillus fragilis. This was later renamed Bacteroides fragilis (Veillon and Zuber) and is the type species of the genus.

Eggerth and Gagnon (1933), in a study of Bacteroides in human faeces, designated the species B. convexus, B. vulgatus, B. distasonis and B. ovatus. These organisms were later regarded as being similar to B. fragilis and were confirmed as subspecies of B. fragilis (Moore and Holdeman, 1973) with the exception of

B. convexus which was shown by Werner (1969) to be identical with B. fragilis and was termed B. fragilis ss. fragilis by Moore and Holdeman (1973). These authors also regarded B. thetaiotaomicron (Distaso, 1912) as a subspecies of B. fragilis and labelled it B. fragilis ss. thetaiotaomicron.

Loesche, Socransky and Gibbons (1964) isolated bacteroides-like organisms from the human mouth; these isolates they termed B. oralis and divided them into two subspecies, B. oralis ss. oralis and B. oralis ss. elongatus. This latter strain was found by Holdeman and Moore (1972) to resemble Fusiformis nucleatus var ochraceus which was first described by Prévot et al., (1956). Holdeman and Moore (1972) re-classified the two organisms isolated by Loesche as B. oralis and B. ochraceus (Prévot), and the terms B. oralis and B. ochraceus have been used in this study.

Bacterium melaninogenicum was the name originally given by Oliver and Wherry (1921) to the organisms they isolated from the mouth, abdominal wounds and faeces and which produced black-pigmented colonies when grown on blood agar. Organisms of this group have been variously classified as Haemophilus melaninogenicus (Bergey's Manual 3rd ed., 1930), Ristella melaninogenicus (Prévot, 1938), Fusiformis nigrescens (Schwabacher, Lucas and Rimington, 1947) and in the 5th edition of Bergey's Manual the currently accepted name of Bacteroides melaninogenicus was proposed by Roy and Kelly (1939). Oliver and Wherry thought the pigment was melanin because of its insolubility in organic solvents, and slow solubility in sodium hydroxide. Schwabacher, Lucas and Rimington (1947) stated that

the black pigment was a derivative of haemoglobin and on examining it spectroscopically found the pattern obtained with the pigment corresponded closely with that obtained with haemin. Tracy (1969) thought that the pigment was colloidal ferrous sulphide. Duerden (1975) demonstrated that when organisms known to produce H_2S were grown in a cooked-meat broth containing cysteine and ferrous sulphate, a colloidal ferrous sulphide was indeed produced. He also found that this was quite unrelated to the black pigment produced by colonies of B. melaninogenicus when grown on blood-containing media. A pigment was extracted from washed cells of B. melaninogenicus and when this was examined spectrophotometrically gave a pattern characteristic of a derivative of haemoglobin. Accordingly, the view of Schwabacher, Lucas and Rimington is sustained and Tracy's generalisation on the pigment of B. melaninogenicus is seen to be misleading. This is of taxonomic importance, as will be discussed later in this thesis.

Sawyer, Macdonald and Gibbons (1962) divided the species B. melaninogenicus into three groups on the basis of biochemical characteristics. These groups were later recognised as subspecies by Cato et al., (1970) and confirmed as B. melaninogenicus ss. melaninogenicus, B. melaninogenicus ss. intermedius and B. melaninogenicus ss. saccharolyticus by Moore and Holdeman (1973).

The organisms originally described as B. corrodens by Eiken (1958) were later found to form two groups: (a) strict anaerobes later classified as B. corrodens, and (b) facultative organisms which were transferred to a new genus Eikenella (Jackson

and Goodman, 1972).

Fusobacterium

The genus Fusobacterium was described by Knorr (1922) who noted three species: F. nucleatum, F. polymorphum and F. plauti-vincentii. Prior to this, a genus Fusiformis was proposed by Hoelling (1910) for spindle-shaped organisms that he observed microscopically but was unable to culture. This genus was accepted in the 1st edition of Bergey's Manual, Bergey et al., (1923), but replaced by the genus Fusobacterium in the 5th edition, Bergey et al., (1939). In the 6th edition of Bergey's Manual (1948), Roy and Kelly designated F. plauti-vincentii as the type strain of the genus. This organism was later considered to be a synonym of F. fusiforme and Leptotrichia buccalis. The name L. buccalis takes precedence, but the genus Fusobacterium is kept in the 8th edition of Bergey's Manual (1974) and Holdeman and Moore propose that a new type species for the genus be approved. Pending a Judicial Commission Opinion, they suggest that F. nucleatum should be designated as the type-species. This organism is now regarded as a synonym of F. polymorphum (Omata and Braunberg, 1960; Hadi and Russell, 1968a). Both names were originally proposed by Knorr (1922) and until a Judicial Commission Opinion is forthcoming both names are valid. The reference strain NCTC 10562 is described in the Catalogue of the National Collection of Type Cultures (1972) as F. polymorphum and this name has been used in the present study.

F. necrophorum originally described by Flügge (1836) was probably the organism described by Loeffler (1884) as "Bacillus der Kalberdiphtherie" (the bacillus of calf diphtheria - a disease with which the organism is still associated). No fewer than twenty-four names have been assigned to this organism in the past (Holdeman and Moore, 1974) and the present name was proposed by Cato et al., (1970).

F. varium (Eggerth and Gagnon, 1933) and F. necrogenes (Weinberg, Nativelle and Prévot, 1937) have both been described and given a variety of names with their current classification being confirmed by Cato et al., (1970).

Leptotrichia

Organisms assigned to this genus are large Gram-negative anaerobic bacilli with one or more pointed ends. The Judicial Commission Opinion 13 (1954) conserved the generic name Leptotrichia (Trevisan, 1897) and designated L. buccalis as the type strain. F. plauti-vincentii and F. fusiforme (as described by Hoffman, 1957) are later synonyms of L. buccalis which are thus invalid names (Holdeman and Moore, 1974). An organism once called L. dentium (and still listed in the Catalogue of the National Collection of Type Cultures, 1972) has been shown to be a Gram-positive, branching filament that will grow aerobically and has been designated Bacterionema matruchotii (Gilmour, Howell and Bibby, 1961).

Further detailed considerations of the historical

classification of these organisms are given by: Sebald (1962), Spiers (1971), Aalbak (1973), and Holdeman and Moore (1974).

Bacteroidaceae as members of commensal flora

Members of the family Bacteroidaceae are found as part of the commensal flora of various sites in man and in a wide variety of animals. In addition, B. serpens has been isolated from heavily contaminated water, F. aquatile from fresh water and B. capillosus from sewage.

There is little available in the literature on the occurrence of Bacteroidaceae in the commensal flora of animals. Holdeman and Moore (1973 and 1974) note two organisms isolated from the rectum of the termite, B. termitidis and B. niger. F. varium isolated from the roach is the only member of the Bacteroidaceae to be reported in a fish. Rodents have been a little more studied and Holdeman and Moore (1973) report F. varium, F. rusii, F. naviforme, B. fragilis ss. distasonis, and B. fragilis ss. thetaiotaomicron as being commensal in the gastro-intestinal tract of rats and mice. These authors also report the isolation of five species of Gram-negative, non-sporing anaerobes from the gastro-intestinal tract of pigs; B. capillosus, B. corrodens, B. fragilis ss. distasonis, B. ruminicola and F. necrophorum.

Considerable attention has been paid to the flora of the caeca of poultry. Gram-negative, non-sporing, anaerobic rods form about 40% of the caecal flora of chickens (Barnes and Impey, 1970).

Various groups of organisms, not all further identified, have been described by Barnes and Impey (1968 and 1970). One of the largest groups contained the organism now known as B. hypermegas (Barnes and Impey, 1971) which is a common commensal in the caeca of chickens, ducks and turkeys. Other organisms found in the caeca of poultry include B. clostridiiformis, B. fragilis ss. ovatus, B. fragilis ss. thetaitaomicron, and F. necrogenes.

Much of the renewed interest in Gram-negative, non-sporing anaerobes arose following developments in techniques that were required to study the flora of the bovine rumen. The techniques developed by Hungate (1950) enabled cellulolytic rumen organisms to be grown in the laboratory in very reduced conditions. A detailed account of the organisms found in the bovine rumen was given by Hungate (1966). Among the Bacteroidaceae listed were B. succinogenes, B. amylophilus, B. ruminicola, Selenomonas ruminatum, Selenomonas lactilytica and Butyrivibrio sp. Other rumen organisms noted by Holdeman and Moore (1974) include B. nodosus, B. clostridiiformis and F. necrophorum. The ecology of the rumen was discussed by Bryant (1963) and Hungate (1966).

Although most studies on animal flora are directly related to pathological conditions, B. melaninogenicus has been reported as commensals in the gingival crevices of beagle dogs (Courant et al., 1968).

In humans the commensal role of the Gram-negative, non-sporing anaerobes has been studied in detail. These organisms

are found in the gut, the female genital tract and in the mouth.

Drasar (1974b) noted that the flora of the gut could be affected by a number of factors: intestinal secretions, intestinal mucosa, immune mechanisms, bacterial interactions, bacterial contamination, diet, and antibacterial drugs. Few bacteria are found in the duodenum or jejunum but considerably more are found in the ileum (see Table 1). The faecal flora is similar to that of the large intestine; the faecal flora of subjects in various parts of the world was surveyed by Drasar (1974b).

B. fragilis is the dominant species in the human gut and has been reported in concentrations of 10^{10} to $10^{11.5}$ per gram wet weight of faeces (Drasar, Shiner and McLeod, 1969). Of the subspecies of B. fragilis, B. fragilis ss. thetataiomicron, ss. distesonis and ss. vulgatus were most common in samples of faeces of subjects living in the United States of America (Ueno et al., 1974). Present in some subjects and in lower numbers were B. fragilis ss. fragilis and ss. ovatus, B. putredinis, B. capillosus and B. furcosus. F. varium, F. necrophorum and F. nucleatum were also reported. Ueno and his co-workers reported (1974) that a Japanese subject living in America and eating a Western diet showed a marked decline in fusobacteria which rose again when a Japanese diet was resumed. A similar change was noted in the number of B. fragilis ss. fragilis recovered but a reciprocal change occurred with B. fragilis ss. vulgatus. Other Bacteroides spp. isolated from faeces include B. oralis, B. clostridiiformis and B. ruminicola (Drasar and Hill, 1974).

TABLE 1

The distribution of *Bacteroides* spp.
in the human alimentary tract*

| Site | Log no. of bacteria per ml |
|----------------|----------------------------|
| Saliva | 2 |
| Jejunum | 2 |
| Terminal ileum | 5.7 |
| Caecum | 7.9 |
| Faeces | 10.5 (per g) |

* from Drasar, Shiner and McLeod (1969)

Bacteroides species have been reported as being commensal in the vagina of 5.4% of patients in a survey of 280 pregnant women (Hurley et al., 1974). Gorbach et al. (1973), however, found Bacteroides species to be the most common anaerobe (57% incidence) isolated from the cervix in their study of 30 subjects. B. oralis was isolated from six subjects, B. fragilis from five. B. capillosus, B. clostridiiformis and two unidentified bacteroides-like organisms were isolated from the remaining six subjects. Holdeman and Moore (1973) also indicated the recovery of B. fragilis ss. thetaiotaomicron and F. mortiferum from the female genital tract. Swenson (1974) supported the view that Gram-negative anaerobes were common commensals in the vagina but did not give details of his results.

Organisms of the family Bacteroidaceae are common commensals of the human oral cavity. This site has been studied in some detail and many of the ecological determinants have been investigated (Hardie and Bowden, 1974; Geddes and Jenkins, 1974). B. melaninogenicus, B. oralis, B. ochraceus, F. polymorphum, B. corrodens, F. girans, F. naviforme and L. buccalis have been isolated from the human mouth (Holdeman and Moore, 1973). General reviews of the oral flora have been given by Burnett and Scherp (1968), Davies (1972) and Bowden and Hardie (1974).

The mouth is a complex ecosystem containing a number of different environments in which different flora develop. Not all these environments are suitable for the establishment of anaerobic organisms. In the young adult mouth the chief environments are: (i) saliva, (ii) mucosa of lips, cheeks and palate, (iii) tongue, (iv) gingival crevice, (v) teeth. The teeth themselves contain a number of environments: (a) occlusal surface and fissures, (b) smooth surfaces (buccal and lingual), (c) interproximal surfaces, (d) cervical. These ecological niches are greatly affected by the "normal" functions of the mouth such as the action of chewing food, drinking, tooth-brushing, talking and the eruption and loss of teeth. The preference of anaerobes for certain environments in the mouth is illustrated in Table 2.

TABLE 2

The cultivable microbial flora
isolated from various sites in the human mouth*

| Organism | Percentage of total cultivable flora from: | | | |
|--------------------------|---|--------|------------------------------|---------------------|
| | Tongue | Saliva | Supra- gingival plaque | Gingival crevice |
| Gram + facultative cocci | 44.8 | 44.6 | 27.0 | 28.8 |
| Gram + anaerobic cocci | 4.2 | 13.0 | 12.6 | 7.4 |
| Gram + facultative rods | 13.0 | 16.6 | 23.8 | 15.3 |
| Gram + anaerobic rods | 7.4 | ND | 18.4 | 20.2 |
| Gram - facultative cocci | 3.4 | 1.2 | 0.4 | 0.4 |
| Gram - anaerobic cocci | 16.0 | 15.9 | 6.0 | 10.7 |
| Gram - facultative rods | 3.2 | 2.3 | ND | 1.2 |
| Gram - anaerobic rods | 8.2 | 4.8 | 10.0 | 16.1 |

* Data pooled from Gordon and Gibbons (1966); Gordon and Jong (1968); Gibbons, Kapsimalis and Socransky (1964); Socransky (1970); Socransky and Manganiello (1971).

ND = Not detected.

Although Gram-negative anaerobic bacilli are frequently detected in saliva it appears that these organisms have been dislodged from their primary site by the action of saliva, the soft tissues and mastication of food. There seems little doubt that the gingival crevice and dental plaque form the primary ecological niches for these organisms (Gibbons, Kapsimalis and Socransky, 1964).

In their review of the changes in the oral flora during life, Socransky and Manganiello (1971) note that at birth there are virtually no organisms present in the mouth. Anaerobic organisms are recovered in very low numbers from the infant up to the first year. Bailit, Baldwin and Hunt (1964) reported that 40% of children aged 5-7 years in their survey yielded B. melaninogenicus from gingival crevice debris. This proportion increased markedly in the 7-9 year age group and reached 100% in the 13-15 year age group. Kelstrup (1966b) reported that B. melaninogenicus occurred only in the gingival crevices associated with permanent teeth. This is probably related to the need for the establishment of favourable oxidation-reduction conditions in the mouth and this will occur more readily in the deeper adult gingival crevice (Socransky and Manganiello, 1971). Oxygen tensions in the mouth have been measured by Kenney and Ash (1969) and Eskow and Loesche (1971). These authors suggest that anaerobes can flourish in the gingival crevice and in dental plaque because of the fall in oxidation-reduction potential (Eh) brought about by the metabolism of facultative organisms. The scavenging of traces of oxygen by these facultative organisms is said to reduce the Eh sufficiently to allow spirochaetes to become established in the gingival crevice. Anaerobic organisms may also depend on the facultative bacteria for nutritional factors (Loesche, 1968). A review of the factors influencing the mouth flora is given by Hardie and Bowden (1974), Geddes and Jenkins (1974), and Newman and Poole (1974); the bacterial flora of dental plaque is described by Socransky et al., (1963), Gibbons et al. (1964), McHugh (1969), and Hardie and

Bowden (1975).

No mechanism of bacterial retention or adhesion is known for the oral bacteroides. No adhesive agents such as those produced by the oral streptococci have been described, and adherence to mucosa has not been demonstrated. Socransky and Manganiello (1971) suggest that bacteroides could be retained mechanically, by being embedded in a sticky plaque matrix or by inhabiting the depths of the gingival crevice.

Few changes in the microbial flora of the adult have been reported associated with the progressive loss of teeth. The oxidation-reduction potential rises in the edentulous mouth and, unless dentures are worn, no hard surface exists for the adherence of dental plaque. It follows, therefore, that the anaerobic flora, which is so strongly associated with the presence of teeth, will be greatly reduced in the edentulous mouth (Rosenthal and Gootzzeit, 1942).

The association of Gram-negative non-sporing
anaerobic bacilli with disease

Organisms of the family Bacteroidaceae are generally regarded as opportunistic pathogens. They are part of the commensal flora of man and many animals and when isolated from lesions they are usually in mixed culture (Smith, 1975). In recent years, as anaerobic techniques have improved, Gram-negative non-sporing anaerobes have been isolated more frequently from clinical specimens

and these organisms are increasingly recognised as potential pathogens. Clear experimental evidence of the pathogenic role of these organisms in a specific disease has been produced in only a few instances.

Disease in animals

Berg and Loan (1975) isolated F. necrophorum and B. melaninogenicus from biopsy specimens from cattle with foot-rot. Furthermore they successfully reproduced similar lesions in healthy cattle by applying a culture of these two organisms to the scarified interdigital skin of the hoof. These two organisms were later recovered in large numbers from these lesions. F. necrophorum has been implicated in the aetiology of calf diphtheria but attempts to reproduce these lesions by deliberate infection of the organism have not been successful (Blood and Henderson, 1974).

Foot-rot in sheep has been attributed to a mixed infection by B. nodosus and F. necrophorum (Smith, 1975; Egerton, Roberts and Parsonson, 1969; Roberts and Egerton, 1969). Heel abscess in sheep (bulbar necrosis) has also been attributed to F. necrophorum in a mixed infection (Roberts et al., 1968).

Peridontal disease in dogs has been attributed to Gram-negative anaerobic bacteria (Catcott, 1968; Triadan, 1973) and the so-called fusospirochaetal complex has been observed in dogs with ulcerative gingivitis (Catcott, 1968).

Biberstein, Knight and England (1968) reported 102 isolations of B. melaninogenicus in mixed culture from

infections of domestic animals. This organism was found largely in carnivores, especially cats, and may be the aetiological agent of the purulent serositis.

Disease in humans

Gram-negative anaerobic bacilli are opportunistic pathogens in a wide variety of human infections. Weiss (1937) studied oral strains of B. melaninogenicus and successfully produced infection or inflammation in mice, rabbits and dogs with cultures of these organisms. He later reported on the pathogenicity of B. melaninogenicus (Weiss, 1943) in surgical infections and noted the foul odour of the wounds. The subsequent extensive literature on post-operative wound infection and abdominal infection has been reviewed by Finegold (1974). B. fragilis was the Gram-negative anaerobe most frequently isolated from the cases reported, with B. melaninogenicus and F. nucleatum also being frequently involved in post-operative wound infections. Most specimens yielded mixed cultures (Spaulding et al., 1971). In a summary of the literature on liver abscess, Finegold (1974) noted that F. necrophorum was the most frequently isolated anaerobe; B. fragilis and various unidentified Fusobacterium species were also commonly encountered.

Willis, Young and Ferguson (1974) reported on a group of nine different infections with Gram-negative non-sporing anaerobes; paronychia, post-operative and abdominal infections, pleuropulmonary infections, post-partum and puerperal infections

and a tubo-ovarian abscess. In most of these cases they noted the production of copious amounts of pus, often foul smelling. Septicaemia and thromboembolic complications may follow infection with Gram-negative, non-sporing, anaerobic organisms and these authors stress the need for prompt surgical drainage of pus and suggest the prophylactic use of antibiotics.

Bacteroidaceae have been isolated from 9.3% of blood cultures in a survey of 112 patients (Chow and Guze, 1974). B. fragilis is the most common species of anaerobe isolated from blood cultures (Fellner and Dowell, 1971). Sonnenwirth (1974) reported personal communications from Werner and Dowell that B. fragilis ss. fragilis was the most commonly encountered subspecies; the other subspecies of B. fragilis were much less frequently isolated. In a study of the literature and an analysis of 23 cases of neonatal bacteraemia, Chow et al. (1974) reported 1.8 cases of anaerobic bacteraemia per 1000 live births but reported only one death. They postulated that the condition was self-limiting in neonates. B. fragilis was again the most commonly encountered organism. In their review of the literature they note a mortality rate of 26% in neonates with anaerobic bacteraemia but Chow and Guze (1974) reported a mortality rate of 43% in adults with Bacteroidaceae bacteraemia from other than obstetric cases. A bacteraemia with organisms of the family Bacteroidaceae has been recorded following tooth extraction in 18 out of 25 cases reported by Crawford et al. (1974).

Bacterial endocarditis may, rarely, be caused by

B. fragilis, F. necrophorum and F. nucleatum (Nastro and Finegold, 1973). These authors noted that although the condition was usually subacute two cases presented as a fulminating disease, with death following within 10 days. Fellner (1974) reported a mortality rate of 29% for all anaerobic endocarditis but noted the particularly poor prognosis if B. fragilis had been the causative organism. Endotoxic shock has been reported as a complication of endocarditis caused by bacteroides organisms (Fellner, 1974) and Bodner, Koenig and Goodman (1970) noted endotoxic shock in 10 out of 39 patients with Bacteroidaceae bacteraemia. Yoshikawa, Chow and Guze (1974) described three cases of disseminated intravascular coagulation associated with bacteraemia caused by bacteroides organisms. Finegold (1974) warned that septic thrombophlebitis associated with bacteroides organisms may be a complication of puerperal sepsis or may follow gynaecological surgery.

Finegold (1974) also listed a wide range of infections of the female genital tract which have been associated with Gram-negative anaerobes. Pregnancy, childbirth, abortion, malignancy and obstetric and gynaecological surgery are usually associated with such infections. Many cases of bacteraemia are related to infection of the female genital tract (Fellner and Dowell, 1971). Clark, Marshall and Ackerman (1974) reported an increasing recognition of the role of Bacteroides species in gynaecological infection and in a survey of 403 cultures containing Gram-negative anaerobic bacilli found that most of the specimens were from the female genital tract. In 78% of cases the cultures were mixed.

In a careful and thorough investigation of 33 women with severe infections of the genital tract, Thadepalli, Gorbach and Keith (1973) recovered anaerobes from all patients. B. fragilis was recovered from 26 patients and B. melaninogenicus from eight. In 12 cases, only anaerobes were isolated - two strains in mixed culture and 10 in pure culture.

Bartlett (1974) stated that anaerobic organisms are well recognised as pathogens of the respiratory tract. In summarising his own work on 197 cases of pleuropulmonary infections (lung abscess, empyema, necrotising pneumonia and aspiration pneumonia) he noted that 83% of samples yielded anaerobes on culture. Clinical observations that gave a clue to anaerobic infection were: a suspicion of aspiration; putrid discharge; insidious onset and prolonged duration of symptoms; pulmonary parenchymal cavitation and empyema.

The predominant organisms isolated from 100 cases studied by Bartlett (1974) were F. nucleatum and B. melaninogenicus. Other Gram-negative anaerobic bacilli isolated included B. fragilis, B. oralis and F. necrophorum. Aspiration was thought to be the route of infection in most cases although this is difficult to justify in the case of B. fragilis which is not a commensal of the oral cavity or upper respiratory tract.

Frederick and Braude (1974) used a surgical procedure for collection of samples from chronically infected sinuses and recorded a heavy growth of anaerobic cocci and Bacteroides spp. Other

infections of the upper respiratory tract are similarly associated with organisms that form part of the indigenous flora. The widespread use of antibiotics has markedly reduced the incidence of such infections as mastoiditis and peritonsillar abscess and there are few reports on the anaerobic bacteriology of these conditions - with the exception of oral and dental disease which are discussed on pages 21-24.

Many infections of the central nervous system are caused by facultative organisms. The most common conditions associated with anaerobic organisms in the CNS are brain abscess and metastatic brain abscess.

The role of Bacteroides species and other members of the commensal gut flora in the aetiology of carcinoma of the colon has been discussed by Tabaqchali (1974) and Drasar and Hill (1974). Organisms present in the gut are capable of producing known carcinogens from endogenous steroids such as bile acids and cholesterol. Carcinoma, particularly of the bladder, has been associated with aromatic amines which are produced in the metabolism of tryptophan by Bacteroides species (Drasar and Hill, 1974).

Oral infection. The role of oral bacteria in focal infection has long been postulated. For many years the theory of focal sepsis was accepted and the oral flora, particularly of dental plaque, incriminated in numerous disorders at sites often quite distant from the mouth. Miller in his treatise "The Micro-organisms of the Human Mouth" (1890), stated:

"It has been established beyond all question that myriads of micro-organisms are constantly present in the human mouth, and that these, under favourable circumstances, are capable of manifesting an action of the utmost significance upon the local as well as general health of the patient. Not alone are they responsible for the vast majority of those diseases of the teeth and contiguous parts which the dental surgeon is called upon to treat, but they also give rise to other local and general disorders of the most severe nature."

Infections of oral tissues with Gram-negative non-sporing anaerobes can be divided into three broad groups: (a) infection of the underlying soft tissues and bone including surgical wound infection; (b) periodontal disease; (c) infection associated with the teeth. Osteomyelitis of the maxilla caused by B. funduliformis was reported by Himalstein (1967), and Monaldo et al., (1974) reported an infection in the mandible with sinus formation which tracked into the neck. Bacteroides spp. were recovered in mixed culture from the sinus. The importance of Bacteroides spp. in mixed infections of the bone and soft tissue of the jaws was stressed by Sharp, Meador and Martin (1974) who report a case involving a mandibular fracture with extensive soft tissue damage from which B. melaninogenicus, Pseudomonas aeruginosa and Actinomyces sp. were isolated. Bacteroides infections of the head and neck are usually localised and rarely present problems in management if adequate surgical drainage can be established (Dormer and Babett, 1972). Improvements in techniques of sampling,

culture and identification have made the clinicians more aware of the role of the Bacteroidaceae in oral infection (Dormer and Babett, 1972; Quayle, 1974; Sims, 1974; Sabiston and Gold, 1974). Khairat (1966) and Crawford et al. (1974) stressed that the post-extraction bacteraemia will usually contain anaerobes (57% reported by Khairat and 92% by Crawford et al.); B. melaninogenicus and F. nucleatum are the most frequently cultured anaerobes under these circumstances.

Periodontal disease. Considerable study has been made of the relationship of anaerobic bacteria to periodontal disease. There is a direct relationship between the extent of periodontal disease and the amount of dental plaque in the mouth. Hardie and Bowden (1974) reviewed a number of the studies on dental plaque and periodontal disease and an extensive review of the subject has been given by Socransky (1970) who remained unclear about the precise pathogenic mechanism; he suggested that lytic enzymes, cytotoxic metabolites or a chronic inflammatory response mediated by allergic phenomena are possibilities.

In their now classical experiments on bacterial mechanisms in periodontal disease, Macdonald, Gibbons and Socransky (1960) demonstrated that subcutaneous lesions in guinea-pigs could only be initiated with oral bacteria if B. melaninogenicus was included in the mixture of organisms injected. Infection could be induced with a minimum of B. melaninogenicus, another Bacteroides species, a motile Gram-negative anaerobe and a facultative diphtheroid. Macdonald (1962)

suggested that spirochaetes were secondary invaders of periodontal lesions - merely multiplying in the greatly reduced conditions present - and he stated that Bacteroides species were the most important organisms in the condition. Macdonald, Socransky and Gibbons (1963) demonstrated a net increase in the numbers of organisms in periodontal disease and described experiments that illustrated the collagenolytic activity of B. melaninogenicus. They concluded that "the primary pathogen in mixed infection produced by the flora of the gingival crevice of man is B. melaninogenicus." Socransky and Gibbons (1965) presented evidence of further animal experiments to show that this organism is more overtly pathogenic than had previously been thought. Hadi and Russell (1968_b, 1969) noted an increase in the numbers of Leptotrichia buccalis in chronic periodontal disease. Newman et al. (1974) reported the isolation of 100 strains of Gram-negative, non-sporing, anaerobic bacilli from the depths of gingival pockets. They could be divided into five groups which did not correspond well with the characteristics of recognised species. The investigation of acute ulceromembranous gingivitis (Vincent's infection) has relied largely on microscopic evidence and very few cultural studies have been made. The role of spirochaetes has been disputed and no clear evidence of their role in the aetiology of this condition has been found (Hardie and Bowden, 1974).

Infection of the dental pulp. The bacteriology of infected pulp and periapical tissues was little understood until the advances in sampling and handling of specimens such as

suggested by Möller (1966) were adopted. His careful methodology resulted in high proportions of positive cultures with a marked rise in the recovery of anaerobes. Bacteriological control of endodontic therapy was thus made possible. Although members of the family Bacteroidaceae were isolated from infected root canals the predominant organisms were Gram-positive rods and cocci. Moore and Russell (1972) isolated fusobacteria from a small proportion of the dental abscesses they investigated. They found that aspirated pus yielded more meaningful results than a swab. B. oralis, B. melaninogenicus and B. ruminicola were, however, isolated by Sabiston and Grigsby (1974) from aspirated pus from dental abscesses. Gram-positive anaerobic rods were the most common organisms isolated from necrotic pulp tissue in intact pulp chambers (Kalowsky, 1974), but Gram-negative anaerobic bacilli were the second most common isolates. A similar study by Wittgow, Sabiston and Segerstrom (1974) yielded Bacteroides or Fusobacterium species in 26 out of 40 necrotic pulps sampled. The work of Kantz and Henry (1974) supports these findings. Bacteroides spp. have been reported in deep carious lesions by Edwardsson (cited in Hardie and Bowden, 1974) from a small percentage of the teeth he studied.

Pathogenic mechanisms

A number of explanations have been put forward for the pathogenicity of various members of the Bacteroidaceae. Although most of the animal infections caused by members of this family of organisms are associated with Fusobacterium species, pathogenic

mechanisms have not been extensively studied. A leucocidin produced by F. necrophorum has been reported (Smith, 1975) and these organisms are also known to have an endotoxin. Endotoxins are known to be produced by members of the Bacteroidaceae and have been studied in: B. fragilis (Hofstad and Kristofferson, 1970); F. necrophorum (Hofstad and Kristofferson, 1971a); and B. melaninogenicus (Hofstad and Kristofferson, 1971b). Most studies of the pathogenicity of these organisms have been carried out on B. melaninogenicus strains. The animal inoculation experiments of Macdonald, Gibbons and Socransky (1960) and Macdonald, Socransky and Gibbons (1963) have already been mentioned.

A number of enzymes produced by B. melaninogenicus have been studied which may have a bearing on their pathogenicity. Weiss (1937) reported on the ability of cultures of B. melaninogenicus to lyse fibrin and noted the marked proteolytic activity of some strains. Production of neuraminidase has been noted by Werner and Müller (1971). Perhaps the enzyme produced by B. melaninogenicus, which has been studied most extensively, is collagenase. Few organisms are known to produce a true collagenase and the association of the organism with disease in sites largely composed of collagen tissue has led some workers to speculate that this is the mechanism of tissue destruction in periodontal disease (Gibbons and Macdonald, 1961). The conditions for demonstrating this effect were given by Gibbons and Macdonald (1961), and Hausmann, Courant and Arnold (1967). In a comprehensive review of collagenase and periodontal disease, Fullmer (1971) pointed out

that the amount of collagenase produced by polymorphonuclear leucocytes, connective tissue cells and alveolar osteoclasts exceeded that produced by bacteria. In periodontal disease there is, however, an increase in collagenolytic activity in the gingival crevice. Heat labile cell-free extracts of a B. melaninogenicus strain known to be collagenolytic have been shown to enhance infection by fusobacteria. There is thus, as yet, no clear indication of the effect on the periodontal tissues of the component of collagenase derived from oral bacteroides.

Haemagglutinating activity has been demonstrated in 29 of 59 oral isolates of B. melaninogenicus by Okuda and Takazoe (1974). These strains also possessed fimbriae but no indication as to the possible pathogenic role of these strains was made. Takazoe, Okuda and Yamamoto (1975) isolated 37 strains of oral B. melaninogenicus which possessed a K antigen. These strains were used successfully to infect experimental animals whereas control strains possessing no K antigen did not infect the experimental animals. The presence of a capsule has been associated with pathogenic strains of B. melaninogenicus (Takazoe, Tanaka and Homma, 1971) and in B. fragilis by Werner (1974).

Cellular hypersensitivity to oral organisms has been induced in guinea-pigs by Okada et al. (1975). These organisms included B. melaninogenicus. Lehner (1975) reviewed the immunological aspects of periodontal disease. B. melaninogenicus and "F. fusiforme" (sic) were shown to stimulate lymphocytes in cases of mild periodontal disease. Little further work on the

immune response to Gram-negative anaerobes has been reported.

The pathogenic properties of B. fragilis are little understood. Werner (1974) noted that B. fragilis ss. fragilis was more frequently isolated from specimens of clinical material than from faeces. B. fragilis ss. vulgatus and ss. thetaiotaomicron were more commonly isolated from specimens of faeces. Smith (1975) supports this view that B. fragilis ss. fragilis is the most pathogenic member of the B. fragilis group. Although no definite pathogenic mechanisms are known for this group of organisms, the factors of possible significance were listed by Werner (1974) as (a) endotoxin; (b) the capsule; (c) neuraminidase; (d) fibrinolysin, and (e) haemolysin. Fibrinolysin activity is not found in B. fragilis ss. thetaiotaomicron or ss. vulgatus but is found in ss. fragilis.

The possible pathogenic role of Bacteroides species with relation to disseminated intravascular coagulation (Yoshikawa, Chow and Guze, 1974) has previously been mentioned. A possible carcinogenic role related to the breakdown of bile acids and tryptophane metabolites (Drasar and Hill, 1974) has been discussed earlier.

Techniques of anaerobic culture

Various methods for the culture of anaerobic bacteria have been proposed. Hungate (1950) advocated the use of gassed roll-tubes. This method has been adopted by Holdeman and Moore

(1973), together with careful anaerobic transport and handling of specimens involving gassing of tubes and the use of pre-reduced anaerobically sterilised media. Gordon, Stutman and Loesche (1971) sampled debris from the gingival crevice into oxygen free tubes and used a Hungate system for culture of the specimens. This gave a better recovery than was possible when the specimens were plated out and incubated anaerobically in a Brewer Jar. A glove-box or anaerobic cabinet has been advocated by Aranki et al. (1969) who used the glove-box to reduce the media for two days prior to use. Drasar (1974a) also recommended the use of a cabinet but coupled it with the use of pre-reduced anaerobically sterilised media. Collee, Rutter and Watt (1971) suggested a standardised method for operating anaerobic jars on the principle of the evacuation and replacement of gas. Residual oxygen was removed by room-temperature catalysts and the incubation atmosphere contained CO₂ (Watt, 1973). It has been shown (Watt, 1973) that the addition of 10% CO₂ to the incubation atmosphere markedly increases both the colony size and the numbers of organisms recovered on solid media. Modifications of their original procedure included the use of three catalyst sachets (Watt, Hoare and Collee, 1973) and of a bacteriological indicator of anaerobiosis (incorporation of a culture of Pseudomonas aeruginosa into the jar (Watt, Collee and Brown, in press). The use of freshly poured solid media was also advocated (Watt, 1972a). In many clinical laboratories a hydrogen and CO₂ gas source is obtained by using a Gas-Pak (BBL) sachet which releases the gases on addition of water; the Gas-Pak consists of borohydride and citrate-bicarbonate contained in foil sachets. This system has

been evaluated by Collee et al. (1972) and Dowell (1972) with some reservations noted by Ferguson, Philips and Tearle (1975).

The tolerance of anaerobic bacteria to gaseous oxygen has been studied by Loesche (1969), Hentges and Maier (1972) and Tally et al. (1975). Bacteroidaceae are only moderate anaerobes; representative test organisms could grow in 2-8% O₂ (Loesche, 1969) and survived exposure to air without loss of viability for up to eight hours (Tally et al., 1975). Various possible mechanisms for the failure of anaerobes to grow in air were given by Hentges and Maier (1972) and this complex topic is further reviewed by Watt, 1972a.

Comparisons of various anaerobic techniques, particularly those of jar techniques with cabinet procedures, have been made by numerous authors (Watt, Collee and Brown 1974; Drasar, 1974a; Rosenblatt, Fallon and Finegold, 1973; Starr, 1974). These comparisons are considered in the Discussion section of this thesis.

Nutritional requirements of Gram-negative,
non-sporing, anaerobic bacilli

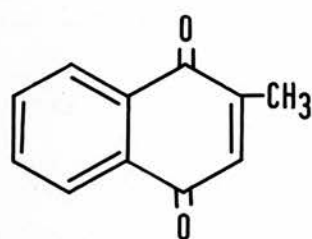
The Bacteroidaceae are quite varied in their nutritional requirements. Some organisms such as B. melaninogenicus and several species of Fusobacterium are much more demanding than B. fragilis. Varel and Bryant (1974) have demonstrated a wide range of biosynthetic mechanisms in B. fragilis which they were able

to grow on fairly minimal defined media. They suggest that this is related to the ecology of these organisms which live in the large intestine and are provided with few nutrients. Consequently the organisms utilize such compounds as haem, vitamin B12 and ammonia which are readily available in the large intestine. Caldwell and Arcand (1974) have shown a requirement by bacteroides organisms for tetrapyrrole compounds and a variety of metallic ions including Fe^{3+} , Mg^{2+} , Ca^{2+} , Co^{2+} and Mn^{2+} . Rumen strains have an absolute requirement for Na^+ .

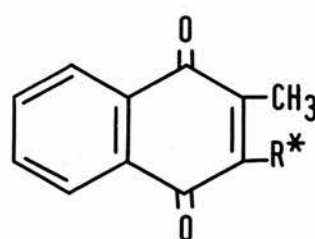
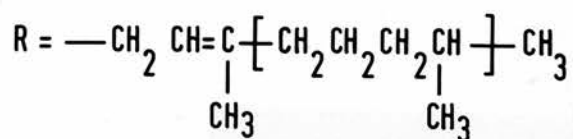
Other growth factors have been determined for B. melaninogenicus strains. The requirement of these organisms for haemin was described by Evans (1951) and by Gibbons and Macdonald (1960). Lev (1958) and Gibbons and Macdonald (1960) found a number of strains of B. melaninogenicus that would only grow on blood agar in the presence of a Staph. aureus contaminant. This dependence on a growth factor from the contaminant organism was removed when menadione (a vitamin K analogue - see figure (i)) was added to the medium. Loesche (1968) postulated that these nutritional requirements would be supplied in the mouth by secretions in the gingival crevice. Rumen strains of B. melaninogenicus have been shown by Lev, Keudell and Milford (1971) to require haemin and vitamin K, and these workers also found sodium succinate to be a growth factor. The incorporation of sodium succinate, haemin and menadione into media gave an increased rate of growth. Sodium succinate could partially replace the requirement for haemin or the requirement for menadione.

Figure (i)

Formula for vitamin K₁ and the
synthetic analogue, menadione



Menadione

Vitamin K₁

MATERIALS AND METHODS

MATERIALS AND METHODS

This study is divided into two main sections: (A) the development of a scheme for the identification of Gram-negative anaerobic bacilli, and (B) the isolation of these organisms particularly from the mouth. Accordingly, the Materials and Methods chapter is here divided into two parts, A and B respectively.

Details of the organisms used in the experiments in Section A are given in Appendices Ia, Ib, and Ic. The media used for studies reported in Section B are described in Appendix II, and the suppliers of materials are listed in Appendix III.

SECTION ATHE DEVELOPMENT OF A SCHEME FOR THE IDENTIFICATION OF
GRAM-NEGATIVE ANAEROBIC BACILLI

Procedure for anaerobic incubation

Anaerobic jars supplied by Baird and Tatlock Ltd (BTL) were fitted with three catalyst sachets. The side-arms of these jars were sealed off. Jars were evacuated to 660 mm Hg below atmosphere pressure and filled with a mixture of 90% H_2 and 10% CO_2 (British Oxygen Company) and the inlets closed. After the jars had stood on the bench for 10 min. one inlet was connected to a manometer and the jar tested for the presence of a secondary vacuum. If a vacuum of 20 mm Hg or greater was recorded, the jar was equilibrated with the H_2 - CO_2 mixture. If the secondary vacuum was less than 20 mm Hg the jar was checked for leaks and for other faults including spent catalysts. This procedure follows largely that of Collee *et al.*, 1972. All incubation was at 37°C.

Incubation in reduced oxygen tension

An anaerobic jar without catalyst was evacuated until the atmospheric pressure in it was reduced to 180 mm Hg. The jar was then refilled with CO_2 to give a final concentration of O_2 and CO_2 of approximately 5% and 80% respectively. Incubation was at 37°C for 48 h.

Maintenance of cultures

Stock cultures were grown in cooked-meat broth (modified from Cruickshank, 1968) for 48 h in an anaerobic jar and stored on the bench. Viability was retained for up to two weeks. Cellular morphology was regularly checked by the examination of Gram-stained smears, and the purity of cultures checked by aerobic and anaerobic

subculture on human-blood agar.

Culture inocula

One drop (0.02 ml) of a 48-h cooked-meat-broth culture was used to seed each tube and either one drop or one loopful (c 0.01 ml) was used to seed each plate of medium.

Organisms

Initially 165 strains of Gram-negative anaerobic bacilli were examined (see Appendix Ia). These were reference strains from culture collections; referred strains from other interested workers; clinical isolates from local hospitals; isolates from normal human faeces; and isolates from gingival material sampled in the Edinburgh Dental Hospital.

Subsequently a further 53 strains from similar sources were studied (see Appendix Ib). Finally 72 strains, largely isolated in the clinical sampling surveys, were examined (see Appendix Ic). Of the organisms studied from this last group, 37 were Bacteroides melaninogenicus isolated from the gingival crevice in the clinical sampling survey (method 1) described on page 67 and 30 strains were Gram-negative anaerobic bacilli isolated in the clinical sampling survey (method 2) described on page 72 .

Media

Liquid media were steamed for 30 min. and rapidly cooled before use. When the medium contained heat-labile substances

(e.g. menadione), these were added after steaming. Solid media were either freshly prepared or prepared the previous day and stored aerobically at 4°C.

Cooked-meat broth (see Appendix II) and thioglycollate medium without dextrose or indicator (BBL) were the basic liquid media to which various additions were made (see below) in order to set up a battery of biochemical tests.

Solid media contained Columbia agar base (Oxoid) and blood agar was prepared with outdated human blood provided by the Regional Blood Transfusion Service, Edinburgh. Each 500 ml of the human-blood preparation contained 2g disodium citrate and 1.7 g dextrose in 70 ml of water added to 430 ml of whole blood; lysed-blood agar was prepared by treating the blood with saponin before incorporating it in the medium (Cruickshank, 1968).

Growth factors

Liquid media not containing cooked-meat particles, and solid media not containing whole blood, required one or more growth factors to support the growth of many of the strains. Haemin was prepared in a stock solution containing 500 µg/ml of haematin hydrochloride (BDH Chemicals Ltd) in 0.01N NaOH. The stock solution of menadione (Sigma, London) contained 100 µg/ml in distilled water (Barnes and Impey, 1971). These solutions were sterilised by membrane filtration and stored in mixed solution at 4°C. This solution was added aseptically to cooled, autoclaved or pre-steamed media to give final concentration of haemin 5 µg/ml and menadione

1 $\mu\text{g/ml}$. A solution of glucose in distilled water, sterilised by membrane filtration, was added to cooled media to give a final concentration of 1%. Yeast extract (Oxoid, 0.25%) and sodium succinate (BDH, 0.25%) were added before autoclaving.

Tests for the characterisation of strains

Colonial morphology. This was usually studied after growth for 48 h on blood agar. Morphological characteristics of organisms that grew slowly were recorded as soon as growth was visible.

Cellular morphology was noted on Gram-stained smears from cultures grown for 48 h, (i) on blood agar, and (ii) in cooked-meat broth.

Haemolysis on blood agar was observed after incubation for 48 h and one week.

Pigment production. Strains were observed for the production of black-pigmented colonies on lysed-blood agar with menadione after incubation for up to two weeks.

Motility. A wet-film prepared from a 48-h cooked-meat-broth culture was examined by phase-contrast microscopy. Strains were also stab-inoculated into semi-solid agar (motility test medium) which was then incubated anaerobically until growth was visible. The medium contained 0.2% agar (Oxoid no. 2) in thioglycollate medium with yeast extract, haemin and menadione.

Lipase activity. Cultures were grown on egg-yolk agar (Cruickshank, 1968) supplemented with sodium succinate, yeast extract, haemin and menadione. Lipase activity was indicated by the production of a pearly-layer effect on the medium.

Oxidase production. A positive reaction was indicated by a blue colour developing in the colony after a freshly prepared 1% solution of tetramethyl-p-phenylene-diamine dihydrochloride in distilled water had been poured on to a one-week culture on supplemented egg-yolk medium.

Catalase production. A one-week culture on supplemented egg-yolk agar was flooded with 10% hydrogen peroxide; a stream of bubbles arose from catalase-producing colonies.

H₂S production. A positive reaction was detected by blackening of a strip of lead acetate paper suspended during incubation in the neck of a tube of cooked-meat-broth culture.

Nitrate reduction. This test was carried out in thioglycollate medium with yeast extract, haemin, menadione and KNO₃ (200 µg/ml). The presence of nitrite ions was indicated by a deep red colour when 0.5 ml of Nitrate Solution A and 0.5 ml of Nitrate Solution B (Cruickshank, 1968) were added to 48-h cultures.

Indole production. 0.5 ml of benzol was added to the liquid supernate of a 48-h cooked-meat-broth culture and mixed. A few drops of Ehrlich's Reagent were added and this reagent turned pink if indole was present.

Gelatinase test. A charcoal-gelatin disk in a cooked-meat-broth culture was observed for digestion of the disk during incubation for two weeks. In view of difficulties encountered with commercially available disks which gave irregular results,

the disks were prepared in our own laboratory by a modification of the method of Kohn (1953): 12.5 g of gelatin (Difco) was dissolved in 100 ml nutrient broth (Oxoid no. 2); 5 g of finely powdered charcoal was added and the mixture was poured into metal Petri dishes to solidify at 4°C. The charcoal gelatin was held in 10% formalin at room temperature for five days and then cut into disks of 1 cm diameter. The disks were washed in running tap water for 48 h at 4°C and pasteurised by heating at 70°C in sterile distilled water for 20 min.

Aesculin hydrolysis. Development of black discolouration on adding 0.5 ml of a 1% aqueous solution of ferric ammonium citrate to a 48-h culture in cooked-meat broth containing 1% aesculin indicated a positive reaction.

Dextran hydrolysis. Strains were grown for one week on a medium containing 0.5% dextran T40 (Pharmacia), 0.5% blue dextran (Pharmacia), 0.2% glucose, 0.25% sodium succinate, 0.25% yeast extract, 5 µg/ml haemin, and 1 µg/ml menadione in Columbia agar base (Oxoid) (modified from Staat, Gawronski and Schachtele, 1973). Hydrolysis was detected by the development of a zone of clearing around colonies growing on the blue medium.

Carbohydrate-fermentation tests. Aqueous 20% solutions of glucose, lactose, sucrose, maltose, rhamnose, trehalose and mannitol were sterilised by membrane filtration. A volume was separately added to a tube of pre-steamed thioglycollate medium with haemin, menadione and yeast extract to give a final

concentration of 1% of the test sugar (modified from Loesche, Socransky and Gibbons, 1964). Series of these tubes were each seeded with one drop (0.02 ml) from a 48-h cooked-meat-broth culture and incubated anaerobically for 48 h. Slow-growing strains were incubated for one week. The final pH was measured with a Pye Unicam model 292 pH meter and a Pye Ingold combined Glass and Reference Electrode no. 401-S/160 after aerobic exposure on the bench for at least 1 h. Controls included cultures of the test strains in sugar-free thioglycollate medium and tubes of uninoculated (but incubated) 1% sugar medium. The test was regarded as positive if the pH fell >0.5 unit below that of the uninoculated control and >0.5 pH unit below that recorded for the inoculated, sugar-free, thioglycollate medium (see Rutter, 1970).

Tolerance tests. In the method finally adopted for these tests, the degrees of inhibition of growth by bile salts and a series of dyes were determined. Strains were grown on each of eight nutrient agar media supplemented with sodium succinate, yeast extract, haemin and menadione, and respectively containing (i) no bile salt or dye (control), (ii) 0.5% sodium taurocholate (BDH), (iii) 0.1% sodium deoxycholate (BDH), (iv) 0.5% sodium taurocholate plus 0.1% sodium deoxycholate, (v) brilliant green (Gurr) (1 in 80,000), (vi) gentian violet (Gurr) (1 in 100,000), (vii) Victoria blue 4R (Gurr) (1 in 80,000), and (viii) ethyl violet (Gurr) (1 in 80,000). The plates were examined for growth after 48 h, or longer for slow-growing strains. The dyes were initially prepared in aqueous solutions and the final concentration in the

plate is given.

Antibiotic-disk-resistance tests. Resistance to 15 antibiotics was determined by the disk-diffusion method on freshly-poured blood-agar plates (modified from Sutter and Finegold, 1971). The plates were seeded by spreading 0.02 ml of a 48-h cooked-meat-broth culture on the surface with a glass spreader. Four disks were placed on each plate and the diameters of inhibition zones were measured after 24 h or 48 h (as soon as good growth was visible).

Disks containing neomycin sulphate 1000 µg, kanamycin sulphate 1000 µg, benzyl penicillin 1.5 units, erythromycin ethyl succinate 60 µg, colistin sulphate 10 µg, rifampicin 15 µg, vancomycin 15 µg, and chloramphenicol 10 µg, were prepared in our laboratory. Those containing methicillin 10 µg, lincomycin 2 µg, clindamycin 2 µg, bacitracin 0.1 unit, neomycin 10 µg, and kanamycin 30 µg were obtained from Mast Laboratories Ltd. Disks containing 5 µg metronidazole were supplied by Dr. J.A. McFadzean of the Research and Development Directorate of May and Baker Ltd. Bacteroides fragilis NCTC 9343 was selected as the reference strain for these tests and a standard Oxford staphylococcus was tested in parallel with it to determine the antibiotic resistance pattern of the reference strain. The pattern obtained with each of the other strains was then compared with the pattern for B. fragilis NCTC 9343.

The inhibition zones were grouped into four grades:

<15 mm (R); 16-25 mm (+); 26-35 mm (++); >35 mm (S). The grades were compared with those obtained with strain 9343 and the strains under test were classified as resistant or sensitive to the particular antibiotic concentration.

Controls

A number of reference strains known to give consistently reliable results were used as control organisms in these tests; both positive and negative controls were used. Media were inoculated with these control organisms and the tests carried out in parallel with those on the test strains. The control organisms for each test and the expected result are given in Tables 3 and 4.

TABLE 3

Control organisms for biochemical and tolerance tests

| Test | Positive control organism | Result | Negative control organism | Result |
|-------------------------------|---------------------------|----------|---------------------------|------------|
| Growth in the presence of: | | | | |
| taurocholate | NCTC 10583 | + | NCTC 9338 | I |
| deoxycholate | NCTC 10583 | I | NCTC 9338 | I |
| taurocholate and deoxycholate | NCTC 10583 | + | NCTC 9338 | I |
| ethyl violet | NCTC 10583 | trace | NCTC 9338 | I |
| Victoria blue 4R | NCTC 10583 | + | NCTC 9338 | I or trace |
| gentian violet | NCTC 10583 | I | NCTC 9338 | I |
| brilliant green | NCTC 10583 | I | NCTC 9338 | I |
| Lipase activity | NCTC 9338 | + | NCTC 9343 | - |
| Oxidase production | <u>Ps. aeruginosa</u> | + | NCTC 9343 | - |
| Catalase production | Oxford staphylococcus | + | NCTC 9343 | - |
| Dextran hydrolysis | NCTC 10582 | ± or + | NCTC 10583 | - |
| Motility | <u>E. coli</u> | + | NCTC 9343 | - |
| H ₂ S production | NCTC 9343 | + | sterile broth | - or trace |
| Indole production | NCTC 10582 | + | NCTC 9343 | - |
| Aesculin hydrolysis | NCTC 9343 | + | NCTC 9338 | - |
| Gelatinase | NCTC 9338 | + | NCTC 9343 | - |
| Fermentation | not done | not done | sterile media | - |
| Nitrate reduction | <u>E. coli.</u> | + | NCTC 9343 | - |

TABLE 4

Grade of zone diameters of control organisms for
antibiotic-disk-resistance tests

| Antibiotic | Zone diameter (mm) after 48 h anaerobic incubation | | |
|------------------------|--|---------------------------------|--|
| | Oxford staphylococcus | <i>B. fragilis</i> NCTC 9343 | <i>B. melaninogenicus</i> NCTC 9338 |
| neomycin 1000µg | + | R | ++ |
| neomycin 10µg | R | R | R |
| kanamycin 1000µg | + | R | R |
| kanamycin 30µg | R | R | R |
| penicillin 1.5 units | S | R | S |
| methicillin 10µg | ++ | R | S |
| erythromycin 60µg | ++ | S | S |
| colistin sulphate 10µg | R | R | ++ |
| rifampicin 15µg | ++ | ++ | ++ |
| lincomycin 2µg | + | R | S |
| clindamycin 2µg | + | + | S |
| bacitracin 0.1 unit | R | R | + |
| vancomycin 15µg | + | R | R |
| chloramphenicol 10µg | + | + | ++ |
| metronidazole 5µg | R | ++ | S |

Tests used to characterise each group of strains

All the tests described in Section A of this chapter were performed on the 165 strains that formed the first group of organisms studied. Certain tests were found to be of good discriminatory value and these were used for the identification of later groups of strains. These tests are noted in Table 5 and were those used in the identification scheme developed (see Duerden, Holbrook, Collee and Watt, 1976 and figure (v)). In order to evaluate the discriminatory value of the tests listed in Table 5 a second series of strains (listed in Appendix Ib) was studied in all the reactions noted in Section A of this chapter. The identities obtained from the results of these tests were noted. Identification was also made using only the tests listed in Table 5 and the results are given in Table 24.

The remaining 72 strains were mainly oral isolates from the clinical sampling surveys. These strains (listed in Appendix Ic) were identified by the series of tests listed in Table 5 and the results are noted in Tables 25 and 26.

TABLE 5

Tests used to identify strains listed in Appendices Ib and Ic which are used in the identification scheme

- 1) Pigment production on blood agar
- 2) Antibiotic-disk-resistance tests:
 - a) neomycin 1000 µg
 - b) kanamycin 1000 µg
 - c) penicillin 1.5 units
 - d) rifampicin 15 µg
- 3) Tolerance tests:
 - a) taurocholate
 - b) deoxycholate
 - c) Victoria blue 4R
 - d) ethyl violet
- 4) Biochemical tests:
 - a) indole production
 - b) digestion of gelatin
 - c) hydrolysis of aesculin
- 5) Fermentation of carbohydrates:
 - a) glucose
 - b) rhamnose
 - c) trehalose
 - d) mannitol

SECTION BTHE ISOLATION OF ORAL GRAM-NEGATIVE ANAEROBIC BACILLI

A preliminary survey of clinical sampling methods

One hundred subjects were employed in the initial clinical sampling survey. They were either clinical dental students or patients attending the periodontal clinic of the Edinburgh Dental Hospital. The samples comprised debris from the gingival crevice of one or more teeth, but the sampling instrument and method of treating the sample varied. Sampling instruments were either Younger-Good curettes or sterile, chisel-edged, wooden sticks (swab-sticks). Samples were either plated directly on to solid medium or conveyed to the laboratory in a transport medium. The methods adopted are shown in Table 6.

TABLE 6

Methods used in the preliminary survey of clinical sampling methods

| Sample no. | Instrument used | Method of treatment of sample | Subject |
|------------|-----------------|--|----------|
| 1-14 | curette | transport in VMG II | students |
| 15-24 | curette | transport in thioglycollate | students |
| 25-34 | curette | transport in VMG II | patients |
| 35-51 | wooden stick | direct plating & transport in VMG II & thioglycollate | students |
| 52-100 | wooden stick | direct plating | students |

Samples were removed by scraping the instrument along the gingival margin with the tip pressed firmly into the gingival

crevice, a technique similar to that used in the scaling of teeth. The sample was either placed on to a lysed-blood-agar plate containing 1 µg/ml menadione (see appendix II) or transferred to 7.0 ml of transport medium in a bijou bottle. Two transport media were employed; VMG II (Møller, 1966) and Thioglycollate medium without dextrose or indicator (BBL). On reaching the laboratory, samples were placed in an ultrasonic bath (Kerrys Ultrasonics Ltd) and the sample dispersed by ultrasonication for 15 min at 80 KHz. One loopful of transport medium (c 0.02 ml) was then used to inoculate a lysed-blood-agar plate containing menadione 1 µg/ml. Anaerobic incubation, according to the method described in Section A, was initially for 48 h at 37°C. At this stage the plates were examined and the presence of black-pigmented colonies recorded. Any such colonies were picked and subcultured. Plates were re-incubated for one week and examined daily for the presence of black-pigmented colonies. Pure cultures of organisms producing black colonies were grown up in cooked-meat broth and lyophilised.

Experiments to develop improved media for use in
further clinical sampling surveys

a) Studies on transport media

Organisms used. Two strains of Bacteroides melaninogenicus ss. intermedius were used in quantitative experiments to evaluate the transport media. One was NCTC 9338 and the other a fresh clinical isolate given the laboratory number WPH 99. This latter strain was

isolated from the direct plating of a subgingival plaque sample on to a lysed-blood-agar plate containing menadione 1 µg/ml.

A discrete black colony was seen which was subcultured on a further lysed-blood-agar plate, and a colony was used to seed a pre-steamed cooked-meat broth and, after 48 h incubation, the organisms were lyophilised.

Pilot counts on standard cultures. Starter cultures of the test strains (NCTC 9338 and WPH 99) were prepared by opening the freeze-dried ampoules into pre-steamed, cooked-meat broth. Purity checks were made by inoculating blood-agar plates with the suspension of lyophilisate. These checks were incubated aerobically and anaerobically. The broths were incubated anaerobically for 48 h at 37°C. Standard cultures were made by inoculating two pre-steamed, cooked-meat broths with 0.1 ml of the respective starter culture and incubating anaerobically at 37°C for 48 h. Dilutions of these broths of 10^2 , 10^3 , 10^4 and 10^5 were then made.

Inocula of 0.02 ml were spread on lysed-blood-agar-menadione plates with a separate sterile glass spreader for each plate. These plates were incubated anaerobically at 37°C for 48 h and colony counts were then made. The plates with approximately 500 colonies were used for counting and the total viable count of the standard culture determined. Total microscopic counts were done with a Hawksley counting chamber (Hawksley, England, 0.1 mm depth, Thoma ruling).



Method. Four transport media were examined for their ability to maintain viability of the two strains of B. melaninogenicus for up to 48 h, viz.: (i) VMG II (Möller, 1966); (ii) thioglycollate without dextrose or indicator (BBL); (iii) Reduced Transport Fluid (Syed and Loesche, 1972); (iv) the diluent used by Gordon, Stutman and Loesche (1971). Formulations of these media are given in Appendix II.

Suitable dilutions of the standard cultures were determined from the results of the viable counts of these standard cultures. The culture of NCTC 9338 was accordingly diluted 1:100 and WPH 99 diluted 1: 200. Dilutions were made with pre-steamed nutrient broth. Inocula of 0.02 ml of each culture were placed in each of the four transport media. The cultures were mixed by rolling the inoculated bijoux bottles. For each transport medium, four lysed-blood-agar plates containing menadione, 1 µg/ml, were inoculated with 0.02 ml of the transport medium per plate. These drops were spread over the surface of the plates with sterile glass spreaders; a fresh spreader was used for each plate. All plates were incubated anaerobically for 48 h at 37°C. Meanwhile, the bijoux of transport media were held anaerobically on the bench and at intervals of 1 h, 2 h, 4 h, 6 h and 24 h, samples of 0.02 ml were taken from each transport medium and spread on lysed-blood-agar plates containing menadione 1 µg/ml, as described above. All plates were then incubated anaerobically for 48 h at 37°C.

Following the inoculation of the transport media,

dilutions of 10^4 and 10^5 were made of the standard culture. Inocula of 0.02 ml of each dilution were then spread on four lysed-blood-agar plates containing menadione 1 $\mu\text{g}/\text{ml}$. These plates were incubated anaerobically at 37°C for 48 h and then colony counts performed. The viable count of the standard culture could thus be determined.

After incubation for 48 h, colony counts were performed on all plates and the recoveries of the two strains of B. melaninogenicus, at the various time intervals, were plotted graphically.

b) Studies on liquid media

Organisms used. A number of strains had been encountered in the initial clinical sampling trial that would not grow in the cooked-meat broth routinely used in the laboratory. These strains, together with some exacting isolates from clinical material, had been grown on solid media in pure culture or in association with a deliberately seeded contaminant organism (see Table 7). The colonies were then removed from the plate and lyophilised. Reconstitution was carried out on lysed-blood-agar plates containing menadione 1 $\mu\text{g}/\text{ml}$ deliberately inoculated with a Staphylococcus aureus contaminant. Inoculation of the contaminant was done as shown in figure (ii), which avoided direct contact of the organisms.

TABLE 7Sources of strains used to evaluate liquid media

| Strain number | Identity and Source* |
|---------------|--|
| 2296 | <u>B. melaninogenicus</u> ss. <u>asaccharolyticus</u> isolated from a gangrenous wound - Bangour Hospital, W. Lothian. |
| 2/2009 | a fusiform organism isolated from a blood culture - Royal Infirmary, Edinburgh. |
| WPH 12 | A staphylococcus-dependent strain of unknown identity isolated from a blood culture - Royal Infirmary, Edinburgh. |
| WPH 32 | A staphylococcus-dependent strain of <u>B. melaninogenicus</u> from a high vaginal swab - Royal Infirmary, Edinburgh. |
| WPH 34 | A staphylococcus-dependent strain of <u>B. melaninogenicus</u> from gross calculus - Edinburgh Dental Hospital. |
| WPH 36 | A staphylococcus-dependent strain of <u>B. melaninogenicus</u> from a dental abscess - Royal Infirmary, Edinburgh. |

Figure (ii)

Method of inoculation of an exacting
strain of *B. melaninogenicus* with
a *staphylococcus* contaminant

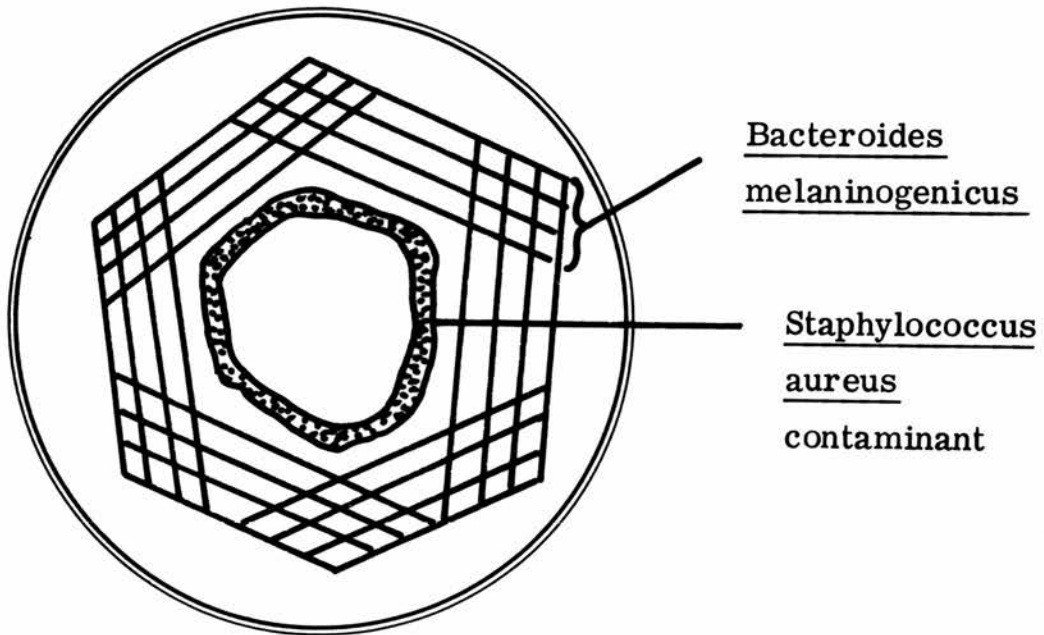


TABLE 7 (continued)

| Strain number | Identity and Source* |
|---------------|--|
| WPH 37 | A staphylococcus-dependent strain of <u>B. melaninogenicus</u> from a high vaginal swab - Royal Infirmary, Edinburgh. |
| WPH 38 | A staphylococcus-dependent strain of unknown identity from a high vaginal swab - Western General Hospital, Edinburgh. |
| WPH 42 | A strain of <u>B. melaninogenicus</u> ss. <u>asaccharolyticus</u> which would only grow on solid media containing blood; isolated from a high vaginal swab - Royal Infirmary, Edinburgh. |
| WPH 43 | A strain of <u>B. melaninogenicus</u> ss. <u>asaccharolyticus</u> which would only grow on solid media containing blood; isolated from a high vaginal swab - Royal Infirmary, Edinburgh. |

* These notes refer to growth problems encountered prior to the development of improved media.

Method. A total of eight media were examined:

- (i) Robertson's cooked-meat broth (Cruickshank, 1968);
- (ii) thioglycollate medium without dextrose or indicator (BBL) but containing haemin, menadione, yeast extract and sodium succinate; (iii) brain-heart-infusion broth (Oxoid) with haemin and menadione; (iv) horse-digest broth with haemin and menadione;
- (v) B.M. broth (Williams, Bowden, Hardie and Shah, 1975);
- (vi) a modification of B.M. broth; (vii) modified medium E (Mr. A. Deacon, personal communication); (viii) NCTC 109 TC medium with haemin and menadione.

The formulation and preparation of these media is given in Appendix II. In order to reduce NCTC 109 TC medium prior to use, it was stored anaerobically overnight at room temperature. Haemin, menadione and L-glutamine were added to this medium immediately prior to use. All other media were prepared in a state which could withstand steaming for 30 min. After steaming, the media were cooled rapidly and additions of heat-labile ingredients made immediately prior to use (see Appendix II).

All broths were inoculated with organisms removed from 48-h cultures on lysed-blood-agar plates containing menadione 1 µg/ml. Incubation was anaerobic for 48 h at 37°C. Growth was detected by a visual turbidity and confirmed by the examination of wet-film preparations with phase-contrast microscopy.

c) Studies on solid media

Organisms used. B. melaninogenicus ss. intermedius

NCTC 9338 and the fresh clinical isolate strain no. WPH 99 were used.

Method. (1). The recovery of B. melaninogenicus ss. intermedius NCTC 9338 was studied on two solid media; (i) lysed-blood agar containing menadione 1 µg/ml, and (ii) modified medium 10 - sucrose-blood agar (Syed and Loesche, 1973). The formulation of the media is given in Appendix II. 0.1 ml of a 48-h culture of the organism in cooked-meat broth was used to inoculate a further pre-steamed cooked-meat broth. This latter broth was incubated anaerobically at 37°C for 18 h. A total microscopic count was made on this standard culture. Twenty plates of each medium were prepared and stored anaerobically at 4°C overnight, in order to reduce the medium. They were allowed to warm up to room temperature, whilst still held anaerobically, before inoculation. The standard culture was diluted 0.5×10^4 times in pre-steamed nutrient broth. Plates were numbered 1-20 for lysed-blood agar medium and 21-40 for the modified medium 10-sucrose-blood agar. Inocula of 0.02 ml of the diluted standard culture were placed on each plate and the drops spread with a separate, sterile, glass spreader for each plate. Spreading of the drops was done alternately on the two media in the order 1, 21, 2, 22, etc. Plates were allocated to one of four jars in a random manner as determined from randomisation tables (Watt, 1972a, see Table 8).

TABLE 8

The random positions of plates in the four jars
used in the study on solid media (experiment 1)

| Jar | top | Position of Plates in Jar | | | | | | | | bottom |
|-----|-----|---------------------------|----|----|----|----|----|----|----|--------|
| 1 | 1 | 40 | 26 | 20 | 33 | 5 | 22 | 9 | 16 | 32 |
| 2 | 21 | 12 | 30 | 18 | 37 | 34 | 15 | 25 | 7 | 2 |
| 3 | 3 | 11 | 38 | 23 | 35 | 31 | 6 | 14 | 17 | 27 |
| 4 | 19 | 28 | 8 | 10 | 29 | 4 | 24 | 39 | 36 | 13 |

The plates were incubated anaerobically at 37°C for 48 h when colony counts were made and the recovery rates of the strain on the two media were determined.

Method (2). The recovery rate of the fresh clinical isolate, WPH 99, was determined on three solid media; (i) lysed-blood agar containing menadione 1 µg/ml; (ii) modified medium 10 - sucrose-blood agar (Syed and Loesche, 1973); (iii) modified B.M.-blood agar (modified from Williams, Bowden, Hardie and Shah, 1975). The details of the preparation of these media are given in Appendix II.

All plates were stored anaerobically at 4°C overnight (in order to reduce the plates); they were allowed to warm up to room temperature, whilst still held anaerobically, prior to inoculation. A 10⁵ dilution in pre-steamed nutrient broth was made of a 48-h cooked-meat-broth culture of B. melaninogenicus

strain no. WPH 99. Inocula of 0.02 ml were used to seed the plates. The three different media were each labelled A, B or C and numbered 1-18. Stacks of three plates, one of each medium, were laid out in numerical order 1-18, but with a random arrangement of plates within the stack of three. The plates were seeded in numerical order and the drops spread in the same order with a separate, sterile, glass spreader for each plate. The plates were distributed randomly among six jars (Watt, 1972a, see Table 9) which were then incubated anaerobically at 37°C for 48 h.

TABLE 9

The random position of plates in the six jars
used in the study on solid media (experiment 2)

| Jar no. | Position of Plates in Jar | | | | | | | | bottom |
|---------|---------------------------|-----|-----|-----|-----|-----|-----|-----|--------|
| 1 | A1 | A8 | C9 | C2 | C15 | B16 | B7 | A14 | B1 |
| 2 | C1 | C8 | B12 | A16 | A10 | C17 | B18 | A3 | B5 |
| 3 | B8 | A12 | A5 | A18 | B6 | C10 | B15 | C4 | C13 |
| 4 | B2 | A6 | C3 | B9 | A15 | A9 | C7 | B14 | C18 |
| 5 | C6 | A17 | C12 | B10 | B3 | A7 | B17 | C16 | A2 |
| 6 | B13 | B11 | C14 | A4 | C5 | B4 | A11 | C11 | A13 |

d) Studies on selective media

Organisms used: these are noted in Tables 10 and 11.

TABLE 10

The organisms from culture collections used in the
studies on selective media and enrichment media

| Organism | Strain no. | Source |
|--|------------------------------|--------------------------------|
| <u>Bacteroides melaninogenicus</u> ss. <u>melaninogenicus</u> | VPI 4196 ATCC 15930 30 | VPI ATCC* Dr. E. Sharpe* |
| <u>Bacteroides melaninogenicus</u> ss. <u>intermedius</u> | NCTC 9336 | NCTC |
| <u>Bacteroides melininogenicus</u> ss. <u>melaninogenicus</u> | NCTC 9337 | NCTC |
| <u>Bacteroides oralis</u> | 7CM | Dr. E. Sharpe |
| <u>Bacteroides</u> sp. | B20-a | Prof. G. Sundqvist |
| <u>Fusobacterium polymorphum</u> | NCTC 10562 | NCTC |

* These organisms were labelled B. oralis but were identified in this work as B. melaninogenicus ss. melaninogenicus (see Holbrook and Duerden, 1974).

TABLE 11

The clinical isolates used in the studies on
selective media and enrichment media

| Organism | Strain no. | Source |
|---|--|--|
| <u>Bacteroides melaninogenicus</u> ss. <u>melaninogenicus</u> | WPH 68 WPH 88 | Subgingival plaque, Edinburgh Dental Hospital |
| <u>Bacteroides melaninogenicus</u> ss. <u>intermedius</u> | WPH 99 | Subgingival plaque, Edinburgh Dental Hospital |
| <u>Bacteroides melaninogenicus</u> ss. <u>asaccharolyticus</u> | 2296) 3502) WPH 89 WPH 41) WPH 45) WPH 94) WPH 113) WPH 114) | Routine clinical material, Bengour Hospital Subgingival plaque, Edinburgh Dental Hospital Routine clinical material, Royal Infirmary and West- ern General Hospital, Edinburgh |

In addition five organisms frequently encountered in samples obtained from the oral cavity were studied in order to demonstrate their inhibition by the selective media chosen. The organisms were taken from stock cultures held in the department, viz.:-

"Streptococcus viridans"

Staphylococcus albus

Corynebacterium xerosis

Neisseria catarrhalis

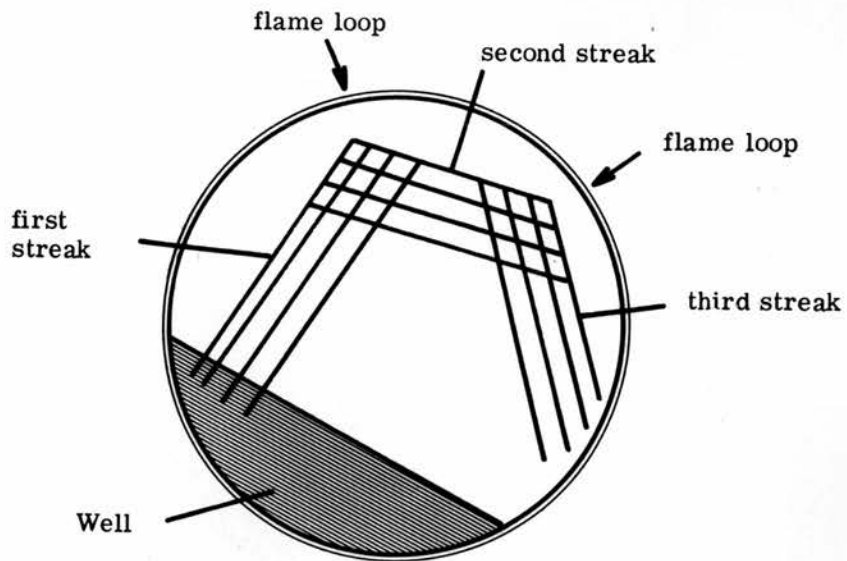
Escherichia coli

Method. The basal medium adopted was modified B.M.-blood agar. This also served as a control. Selective media contained a) 75 µg/ml kanamycin, or b) 75 µg/ml kanamycin plus 2.5 µg/ml vancomycin (see Finegold, Sugihara and Sutter, 1971). Details of the preparation of these media are given in Appendix II.

Standard 48-h cultures of the anaerobic organisms in pre-steamed cooked-meat broth were used. "Streptococcus viridans", Corynebacterium xerosis and Neisseria catarrhalis were grown aerobically for 48 h in Todd-Hewitt broth. Nutrient-broth-cultures of Staphylococcus albus and Escherichia coli were incubated aerobically for 48 h. Inocula of 0.02 ml of each culture were seeded on to both selective media and on to the basal-medium control. The drop of culture was streaked over one third of the area of the plate to form a well and then streaked out across the plate in a standard manner as shown in fig. (iii). All plates were incubated anaerobically for 24 h at 37°C. The degree of growth recorded depended on the extent of growth in the streaking out (see fig. (iii) and "Results" section). All plates were reincubated anaerobically and examined daily for up to five days.

e) Studies on enrichment media

In samples where few Bacteroides melaninogenicus are to be found these organisms may be overgrown by the other bacteria in the sample. An enrichment medium was developed which contained selective agents (antibiotics) in modified B.M. broth with

Figure (iii)Degree of growth:

| | |
|---------------|-----|
| Well only | + |
| first streak | + |
| second streak | ++ |
| third streak | +++ |

Standard method of plating out
fluid inoculum used to determine
the degree of growth on selective media
and from enrichment media.

cooked-meat particles. It was hoped that this medium would allow oral Bacteroides to grow in preference to the other organisms obtained in the sample.

Organisms used. The list of organisms used in the evaluation of the enrichment medium is the same as that used for the selective medium (see Tables 10 and 11).

Method. The formulation of the medium is given in Appendix II. Prior to use, the bottles were incubated anaerobically overnight at 37°C as a sterility check. Immediately prior to use, the haemin, menadione and L-cysteine HCl were added to the medium. Bottles were seeded each with an inoculum of 0.02 ml of 48-h cultures of each organism. This inoculum was mixed through the enrichment broth by gentle rolling of the bottle on the bench. A standard loopful of each enrichment broth (0.03 ml) was used to seed a modified B.M.-blood-agar plate. Wet film preparations were made of each of the cultures used for inoculation, and examined for growth by phase-contrast microscopy. The plates were incubated anaerobically for 48 h at 37°C and, together with the wet-film preparations, served as viability checks of the cultures used for inoculation.

After anaerobic incubation of the enrichment broths for 48 h one standard loopful (c 0.03 ml) was used to inoculate a modified B.M.-blood-agar plate. A wet-film preparation was also made from each enrichment broth and viewed by phase-contrast microscopy and growth recorded. The plates were incubated anaerobically for 48 h at 37°C with the exception of those seeded

with the aerobic organisms which were incubated aerobically at 37°C for 48 h (24 h for E. coli and Strept. viridans). The plates were then examined and the degree of growth recorded.

Clinical sampling survey

This survey incorporated some of the improved media evaluated on the bench in previous experiments.

Media

The solid medium used in this survey was modified B.M.-blood agar with two selective media having (i) 75 µg/ml kanamycin, and (ii) 75 µg/ml kanamycin and 2.5 µg/ml vancomycin incorporated into this basal medium. McCartney bottles containing 20 ml of the enrichment medium were also used and the transport medium selected was VMG II. Details of the preparation of these media are given in Appendix II.

Method (1)

Samples were collected, in batches of five, from 40 clinical dental students and 10 patients attending the periodontal clinic of the Edinburgh Dental Hospital. On the day before the clinical session, non-selective and selective solid media were prepared and stored anaerobically at room temperature. To the enrichment media L-cysteine HCl was added from sterile stock solution to give a final concentration of 0.075%. From the antibiotic stock solutions, kanamycin and vancomycin were added to give final concentrations of 75 µg/ml and 2.5 µg/ml. A stock solution of haemin and menadione was used to give final concentrations of these additives in the enrichment broth of 5 µg/ml and 1 µg/ml respectively. The enrichment broths were then incubated anaerobically at 37°C overnight as a check on sterility.

VMG II transport media were stored aerobically at room temperature in 2.0 ml vials which had had their screw-caps tightened immediately on removal from the autoclave.

In order to reduce the exposure of these media to the air, the anaerobic jars were opened at the chairside. The sampling instrument was a sterile narrow wooden stick with a chisel-edge. Samples were removed with this instrument from the gingival crevice of one or more teeth by an action resembling the scaling of teeth. The degree of periodontal disease present in the area sampled was noted and scored using the Russell Index (as cited by Macphee and Cowley, 1975; see Table 12).

TABLE 12

The degrees of disease denoted by Russell's Index

| Score on Russell's Index | Degree of disease |
|--------------------------|---|
| 0 | no inflammation, no gingival pockets |
| 1 | limited oedematous gingivitis |
| 2 | limited fibrous gingivitis |
| 4 | early periodontitis with true pocket formation |
| 6 | established periodontitis with pocket depth >3 mm but $<$ half the length of the root |
| 8 | severe periodontitis with pocket depth $>$ half the length of the root |

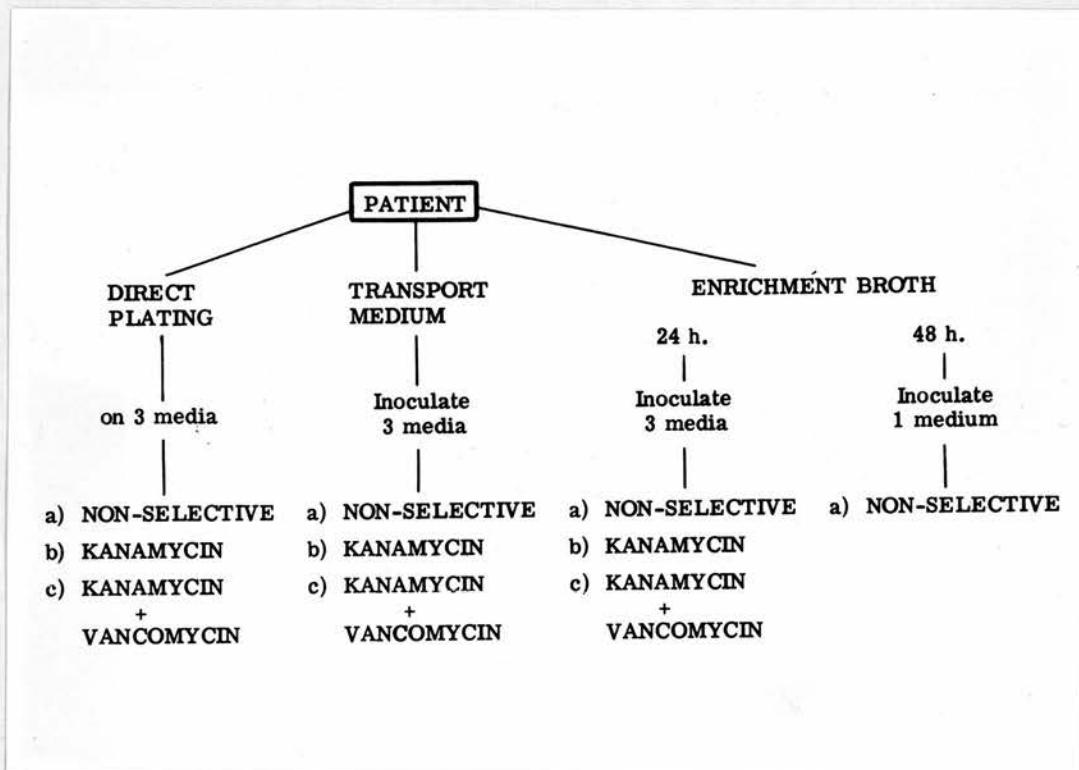
Five samples were taken from each subject, see figure (iv). The first three were plated directly at the chairside on to a non-selective and the two selective solid media; a fourth sample was placed in the enrichment broth and the last sample placed into the transport medium. The inoculated media were then returned to the laboratory where the transport media were placed in an ultrasonic bath (Kerrys Ultrasonics Ltd., model KB 80/2 with generator KG 80/2) and the sample dispersed by ultrasonication at 80 KHz for 15 min.

Meanwhile the samples on the solid media which had been streaked out to form a well were further plated out in the laboratory and incubated anaerobically at 37°C. The enrichment broths, with their screw-caps loosened, were also incubated anaerobically at 37°C. One drop (0.02 ml) of each sample in transport medium was used to seed each of the selective media and the non-selective medium. These drops were streaked out across the surface of the agar in a standard manner and the plates

(Continued on page 71)

Figure (iv)

Diagram to illustrate the procedures adopted for the treatment of samples obtained in the clinical sampling survey



incubated anaerobically at 37°C. The transport media were then discarded.

After incubation for 24 h, the enrichment broths were removed from the anaerobic jar and the bacteria resuspended by gently rolling the bottle on the bench. Inocula of 0.02 ml from each broth were placed on both selective media and the non-selective medium. These drops were spread by a standard plating procedure. The plates had been previously stored anaerobically at room temperature overnight and after inoculation were incubated anaerobically at 37°C. The enrichment broths were reincubated anaerobically at 37°C for a further 24 h; subcultures were then again made in a similar manner but only on to non-selective modified B.M.-blood agar which had been stored anaerobically overnight. The enrichment broths were then discarded and the seeded plates incubated anaerobically at 37°C.

All plates were examined after incubation for 48 h and again at further intervals of 24 h for up to five days. The appearance of black or brown colonies was noted and an attempt was made to subculture such colonies as soon as possible on to anaerobically stored, non-selective, modified B.M.-blood agar. All pure cultures were checked microscopically to confirm that the organisms were Gram-negative bacilli before aerobic and anaerobic subculture. Growth only on the anaerobically incubated plate suggested a presumptive identification of Bacteroides melaninogenicus. Further identification of at least one isolate from each patient was made using the tests in the shortened

identification scheme described in Section A.

For each subject there were ten possible routes by which a colony of B. melaninogenicus could be isolated (see fig. (iv)). The presence of black-pigmented colonies in any one method was recorded. If the colony was eventually isolated in pure culture this was noted. Those occasions when the organism did not grow on subculture, after appearing in mixed culture, and the instances in which no black-pigmented colonies appeared, were noted for each route of isolation. The total number of cases in which pure cultures were obtained; the number in which the organism died on subculture; and the number of cases in which no black colonies were seen, were calculated for each method of isolation. A histogram of these data was prepared. The results of the attempted isolation by each route for each individual subject were drawn up in a table; and this table, and the results as shown in the histogram, were submitted for statistical analysis to the Department of Statistics of the University of Edinburgh (see "Results" chapter).

Method (2)

In order to study the range of organisms that could be recovered by the technique adopted, five samples of subgingival plaque, all taken from different subjects, were held in transport media, returned to the laboratory and sonicated as described in Method (1).

These samples were diluted ten-fold and one-hundred-fold in VMG II transport medium. Three plates of non-selective,

modified B.M.-blood agar and three plates of the selective medium containing kanamycin and vancomycin were inoculated for each dilution. The plates were stored anaerobically at room temperature overnight before inoculation; one drop (0.02 ml) was used to seed each plate and the drops were spread with a sterile glass spreader. Three plates of selective and three of non-selective media were similarly inoculated with undiluted transport medium and the drop streaked out with a bacteriological loop in a standard manner. All plates were then incubated anaerobically at 37°C for three days.

After incubation all colony types were examined and described on any plates showing good growth of discrete colonies. Each variety of colony was Gram-filmed and all colonies of Gram-negative organisms were subcultured anaerobically and aerobically for 48 h at 37°C. All Gram-negative anaerobic bacilli were then grown up in a modified B.M. broth for 48 h and lyophilised. At a later date these isolates were reconstituted and identified according to the shortened scheme for identification as described in Section A.

RESULTS

RESULTS

The results chapter is divided into Sections A and B. Section A here records the results of experiments described in the Materials and Methods Section A in the course of the development of a scheme for the identification of Gram-negative, non-sporing, anaerobic bacilli. Section B relates to the experiments described in Materials and Methods Section B for the development of improved media for the isolation of Bacteroides melaninogenicus and the evaluation of these media in a clinical survey.

Section A

THE DEVELOPMENT OF A SCHEME FOR THE
IDENTIFICATION OF GRAM-NEGATIVE ANAEROBIC BACILLI

All organisms listed in Appendices Ia, b and c were studied by four approaches, morphology, biochemical tests, tolerance tests and antibiotic-disk-resistance tests. Those organisms listed in Appendix Ic were only examined in the tests listed in Table 5. The results of the tests on the 165 strains listed in Appendix Ia have been published by Duerden, Holbrook, Collee and Watt (1976).

Morphology

With two exceptions all of the strains were Gram-negative, non-sporing, obligately anaerobic bacteria. Many strains were highly pleomorphic and the microscopic appearance varied with the culture medium. Cell shape varied from filamentous to cocco-bacillary, often in the same smear. Some strains formed chains, some had pointed ends and others had rounded ends. Cellular morphology had little discriminatory value but certain general points were noted.

(i) B. fragilis strains (plate I) were seen as short bacilli with rounded ends sometimes occurring in short chains.

(ii) B. melaninogenicus strains (plates II and III) were cocco-bacillary sometimes forming short chains; no clear distinction between subspecies was possible on the basis of microscopic appearance. A bizarre morphology including L-forms was observed in a number of exacting strains on the primary isolation plate (see plate IV). The cellular morphology usually reverted to a more

normal appearance on subculture.

(iii) B. oralis strains (plate V) were also cocco-bacillary but were generally smaller than B. melaninogenicus strains. Occasional longer forms were observed in smears of pure cultures of B. oralis.

(iv) F. necrophorum strains (plate VI) were highly pleomorphic bacilli with many long filaments.

(v) F. polymorphum strains (plate VII) had a more classical fusiform appearance; bacilli with pointed ends and occurring singly or in pairs.

(vi) L. buccalis strains (plate VIII) were stout bacilli often with one pointed end and occurring singly or in pairs.

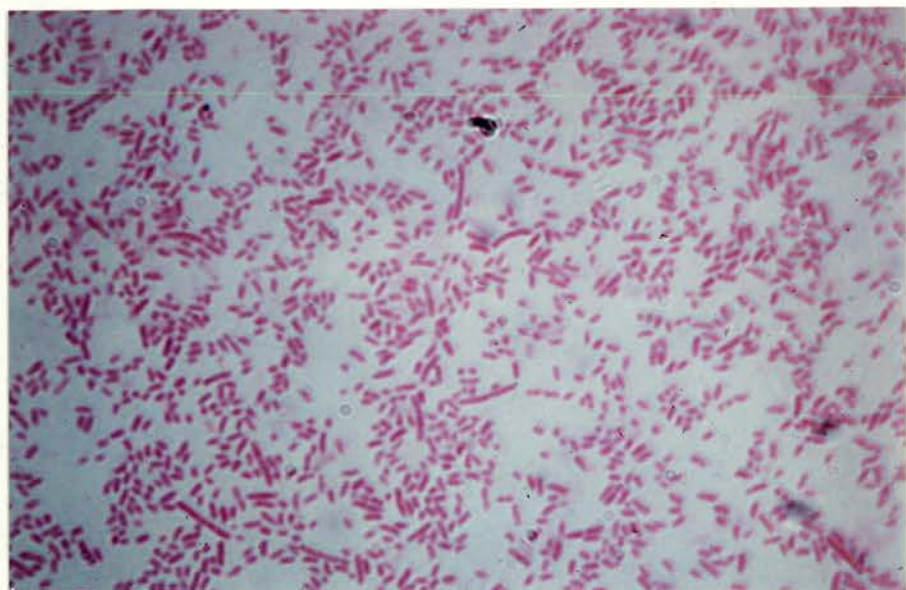
An exception to these general appearances was Leptotrichia dentium NCTC 10206 which was Gram-variable with many Gram-positive cells in young cultures, and was facultatively anaerobic.

B. ochraceus VPI 2845 was found to be strictly anaerobic when first cultured but after repeated subculture it was found to grow in 10% CO₂ in air. This phenomenon is consistent with the description of the organism's behaviour given in Bergey's Manual (Holdeman and Moore, 1974).

Colonial morphology on blood agar was also variable. Colonies differed in size from pin-point to 3-4 mm diameter. Certain colonial appearances were, however, of discriminatory value notably the black-pigmented colonies produced by strains of

Plate I

Microscopic appearance of *B. fragilis* NCTC 9343
grown on blood agar

Plate II

Microscopic appearance of *B. melaninogenicus* ss. *intermedius*
NCTC 9338 grown on blood agar

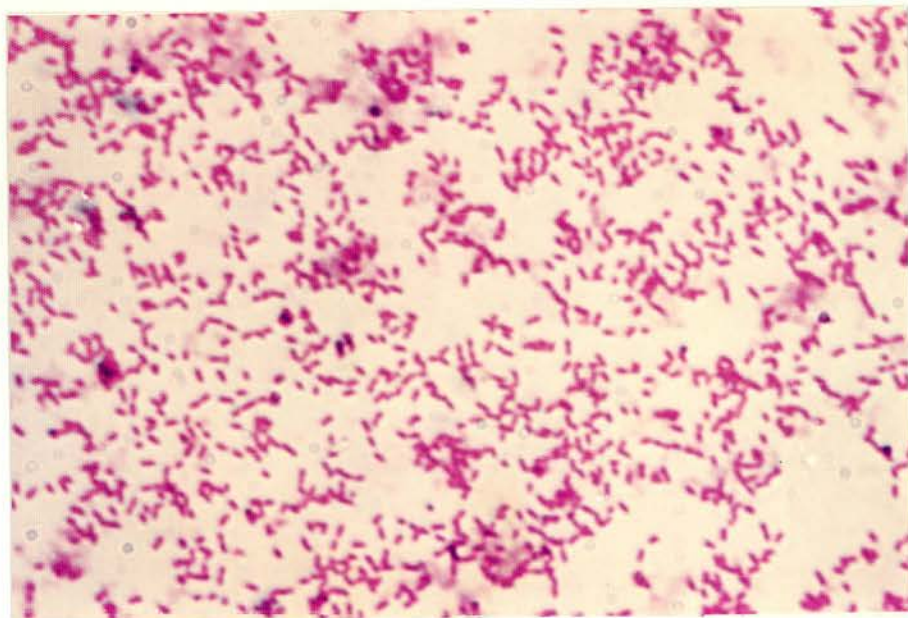
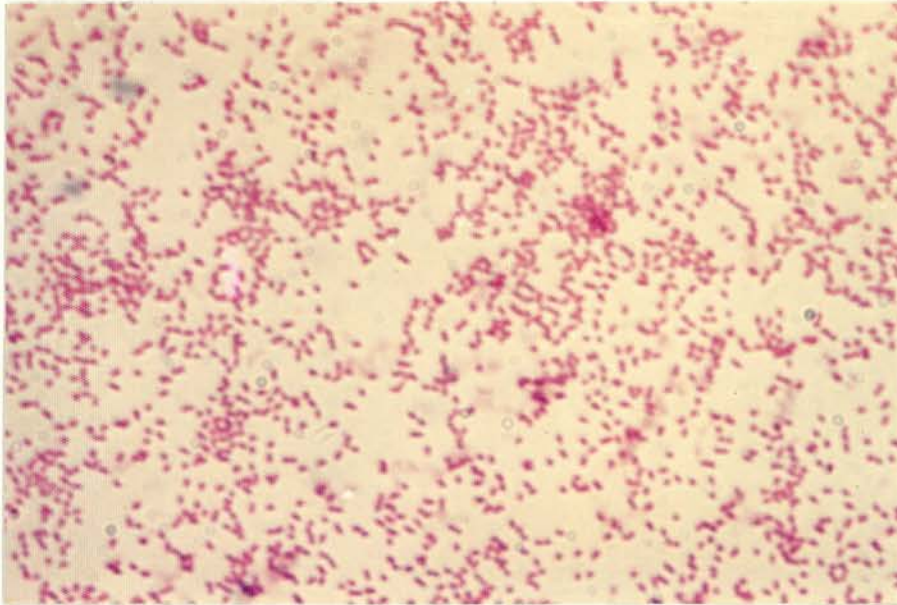


Plate III

Microscopic appearance of *B. melaninogenicus* ss. *melaninogenicus*
ATCC 15930 grown on blood agar

Plate IV

Microscopic appearance of *B. melaninogenicus* WPH 92
showing bizarre morphology on primary isolation

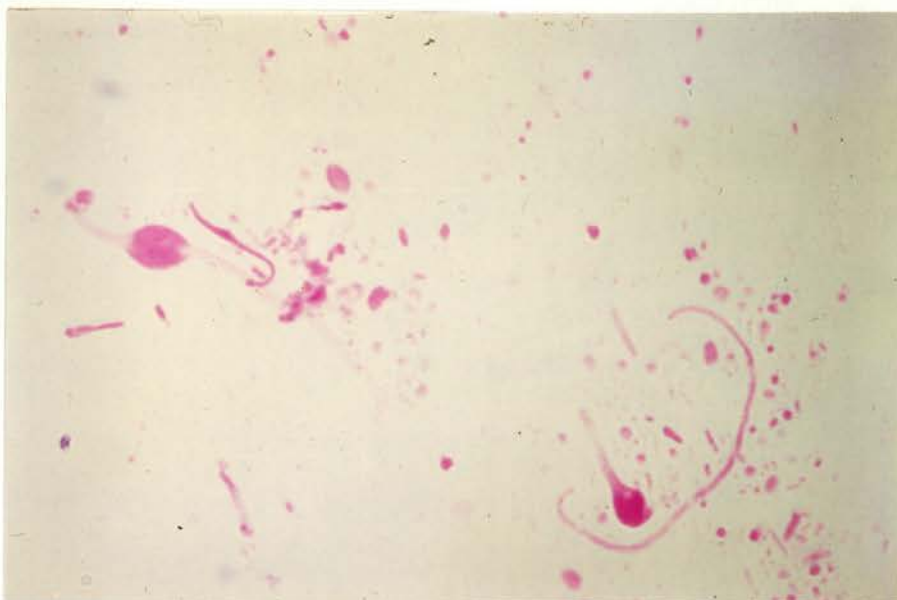
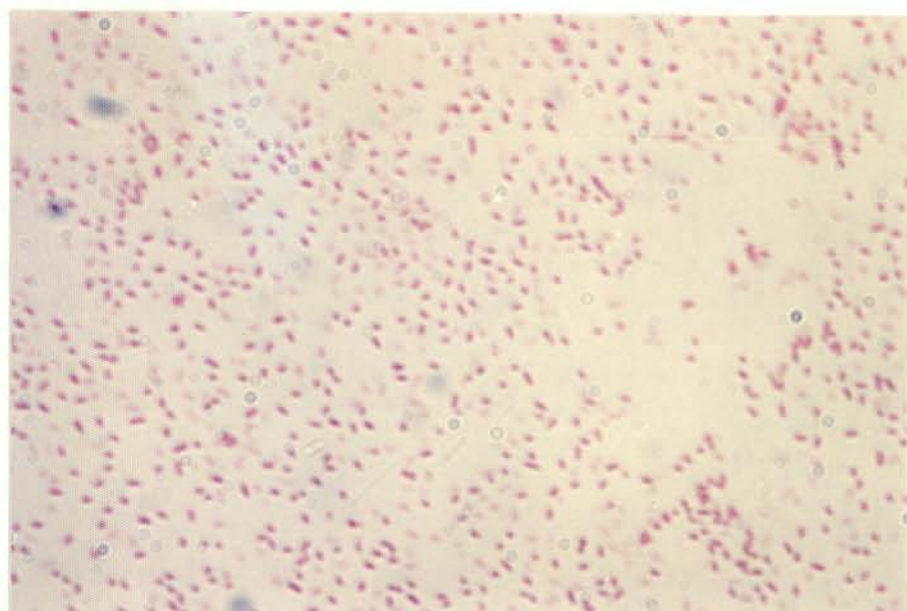


Plate V

Microscopic appearance of *B. oralis* NP 333 grown on blood agar

Plate VI

Microscopic appearance of *F. necrophorum* NCTC 10575
grown on blood agar

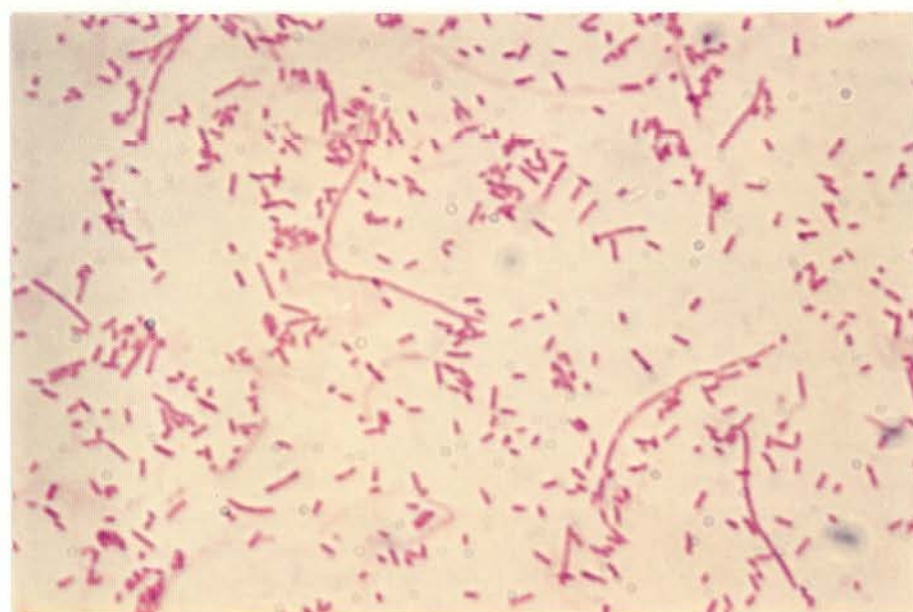
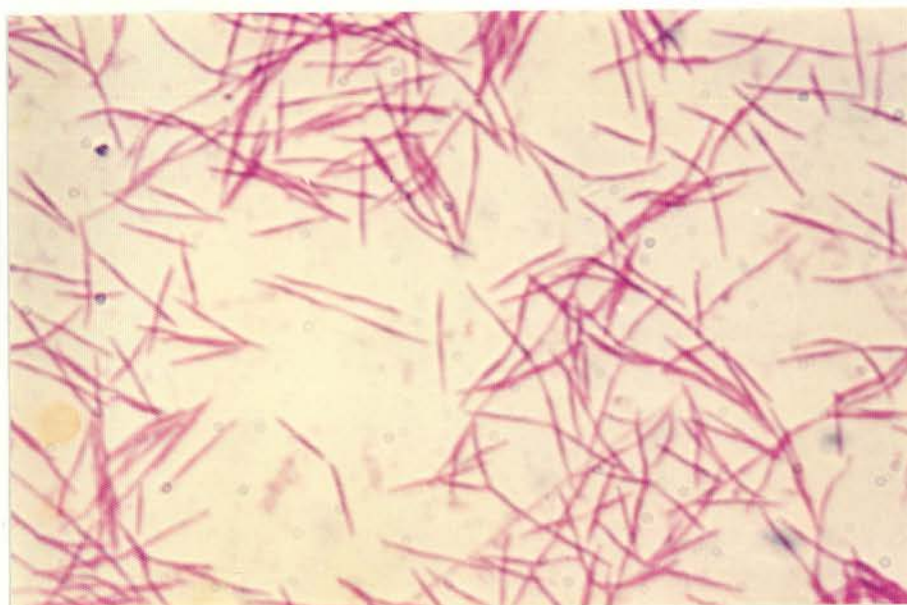


Plate VII

Microscopic appearance of *F. polymorphum* NCTC 10562
grown on blood agar

Plate VIII

Microscopic appearance of *L. buccalis* NCTC 10249
grown on blood agar



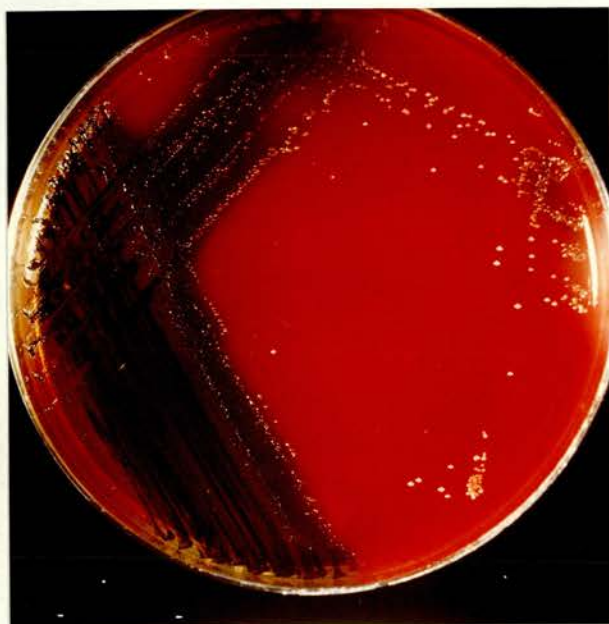
B. melaninogenicus grown on media containing blood (see plates IX and XI). It was observed that typical colonies of B. melaninogenicus ss. melaninogenicus were brown rather than black and on modified B.M. blood agar the colonies of this subspecies were pale with a brown centre (see plate XVI). The production of black-pigmented colonies was speeded up by the replacement of whole blood by lysed blood in the medium (see plates IX and X). Black-pigmented colonies were produced by the strain of B. oralis obtained from the American Type Culture Collection ATCC 15930 (see plate XII) and it was proposed that this strain be reclassified as B. melaninogenicus (see Holbrook and Duerden, 1974).

Some strains of fusobacteria, B. oralis, B. corrodens and B. ochraceus produced rhizoid colonies but not all strains in these groups possessed this property. The pitting of the agar surface, said to be characteristic of B. corrodens, was observed. This feature was often lost on subculture if seen on primary isolation and pitting was sometimes observed with strains of fusobacteria.

Colony variation was noted in pure cultures of certain strains of a number of species; some pure strains of B. melaninogenicus produced large and small colonies on the same plate; B. oralis often produced a rhizoid, ochre-coloured colony which sometimes changed, on subculture, into a smooth, round, blue-grey colony form; and many B. corrodens and Fusobacterium spp. strains also exhibited the rhizoid and smooth colony variation.

Plate IX

Colonial appearance of *B. melaninogenicus* ss *intermedius*
NCTC 9338 grown on blood agar for 48 h to show early
pigment production

Plate X

Colonial appearance of *B. melaninogenicus* ss. *intermedius*
NCTC 9338 grown on lysed-blood agar for 48 h to show the
more rapid development of pigment



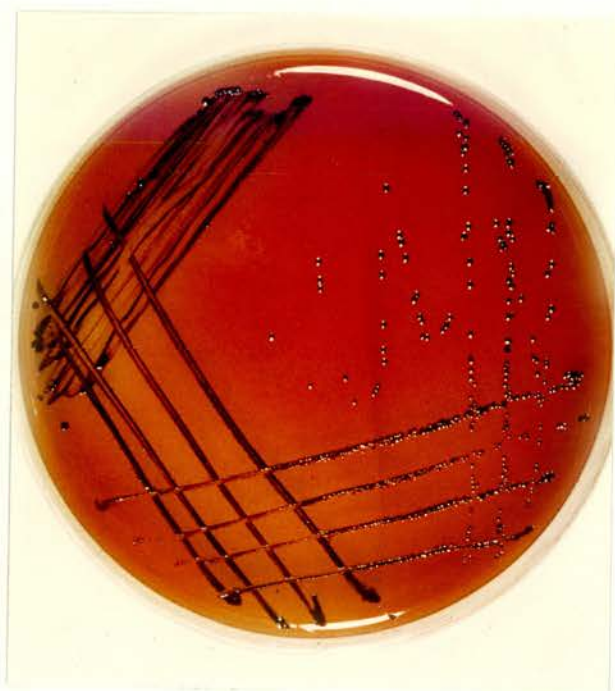
Plate XI

Colonial appearance of *B. melaninogenicus* ss. *intermedius*
NCTC 9338 grown on lysed blood agar for 48 h



Plate XII

Colonial appearance of *B. melaninogenicus* ss. *melaninogenicus* ATCC 15930 (labelled *B. oralis*) to demonstrate the production of black-pigmented colonies by this strain (see Holbrook and Duerden, 1974).

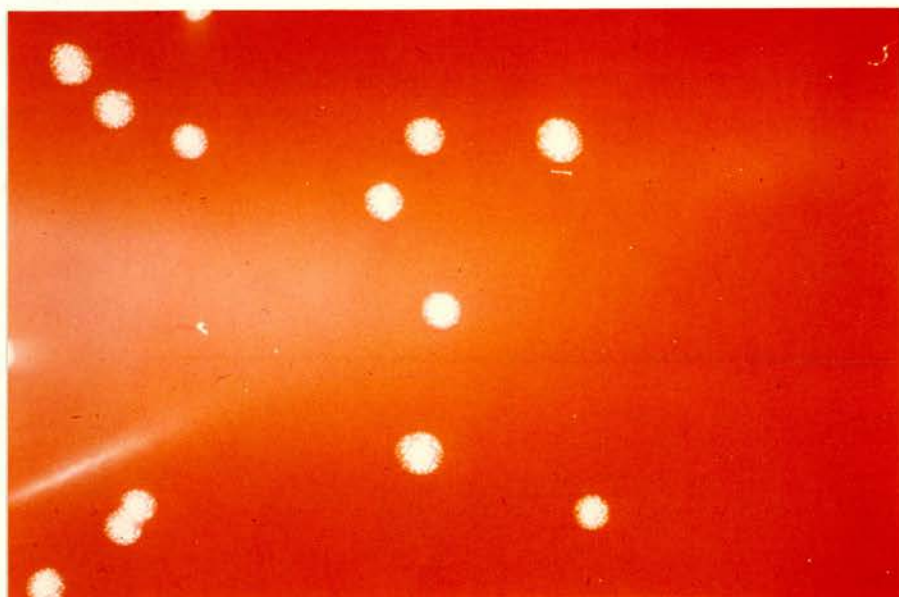


F. polymorphum strains produced small, round, convex, smooth colonies on blood agar (see plate XIII) whereas the colonies of L. buccalis were larger, round convex and granular (see plate XIV). In older cultures colonies of L. buccalis NCTC 10249 tended to produce a rough colony with a raised centre and irregular flat periphery.

Strains of B. melaninogenicus produced a zone of complete haemolysis on blood agar and F. necrophorum was characterised by a wide zone of complete haemolysis. Other organisms produced either no haemolysis or narrow zones of incomplete haemolysis after prolonged incubation.

Plate XIII

Colonial appearance of *F. polymorphum* NCTC 10562
grown on blood agar for 48 h

Plate XIV

Colonial appearance of *L. buccalis* NCTC 10249
grown on blood agar for 48 h



Results of tests on strains listed in Appendix Ia

The tests used in these experiments (Section A) fell into three groups; biochemical, tolerance and antibiotic resistance. All the organisms in the first group tested (see Appendix Ia) were arranged in groups which gave identical patterns of results in each of the groups of tests. For the purpose of analysis the 165 strains listed in Appendix Ia were also arranged into three further groups on the basis of their reactions in the biochemical, tolerance and antibiotic-resistance tests and on their basic morphological characteristics. Those strains which broadly resembled B. fragilis NCTC 9343 were designated "B-strains". The organisms which produced black pigment on blood agar were identified as B. melaninogenicus (Holbrook and Duerden, 1974; Holdeman and Moore, 1974; Duerden, 1975) and designated "M-strains". The remaining strains included a number of reference strains of Fusobacterium and Leptotrichia and a number of isolates which did not conform with the patterns of results obtained for strains in the other two groups.

Results of biochemical tests

The results of the biochemical tests performed on the 165 strains listed in Appendix Ia are given in Tables 13, 14 and 15. These results and tables are extracted from Duerden, Holbrook, Collee and Watt, 1976.

"B-strains". The results of the biochemical tests performed on these 107 test strains are given in Table 13. Twenty patterns of results were obtained with these strains (Bi-xx).

Although most results were either positive or negative, the majority of strains produced only weak or incomplete haemolysis and five strains (patterns Bx and Bxiii) gave variable and weak reactions in the sucrose fermentation test. The five reference strains of B. fragilis ss. fragilis and 47 isolates from clinical specimens gave an identical pattern (Bi).

B. fragilis ss. vulgatus NCTC 10583 and four laboratory isolates, 2 from normal human faeces and 2 from clinical specimens, gave a pattern (Biii) distinguished by the ability to digest gelatin and ferment rhamnose. Pattern Biv, obtained with 12 strains from normal human faeces, differed from this reference pattern only in the inability to hydrolyse aesculin and so these strains were also regarded as B. fragilis ss. vulgatus. The reference strain of B. fragilis ss. vulgatus ATCC 8482 was significantly different; it produced indole and fermented trehalose. This pattern (Bviii) was similar to that obtained with B. fragilis ss. distasonis ATCC 8503 (Bvii) except in the production of indole. B. fragilis ss. ovatus ATCC 8483 (pattern Bxi) was the only strain that fermented mannitol.

The two reference strains of B. fragilis ss. thetaitaomicron ATCC 8492 and NCTC 10582 together with eight clinical isolates and one strain from normal human faeces gave two patterns; Bxiv and Bxv. These were characterised by the production of indole, fermentation of rhamnose and trehalose and inability to digest gelatin. The two reference strains differed

TABLE 13

The results of biochemical tests on B-strains

| Test | Pattern of results* | | | | | | | | | | | | | | | |
|---|---------------------|------------|------------|------------|------------|------------|------------|------------|------------|------------|------------|------------|------------|------------|------------|------------|
| | Bi | Bii | Biii | Biv | Bv | Bvi | Bvii | Bviii | Bix | Bx | Bxi | Bxii | Bxiii | Bxiv | Bxv | Bxvi |
| H ₂ S production | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| Nitrate reduction | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| Haemolysis on blood agar | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| Lipase production on EYA | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| Digestion of gelatin | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| Indole production | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| Hydrolysis of aesculin | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| Fermentation of: | | | | | | | | | | | | | | | | |
| glucose | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| lactose | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| maltose | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| sucrose | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| rhamnose | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| trehalose | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| mannitol | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| Hydrolysis of dextran | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| Reference strains conforming to the given pattern | NCTC 9343 | NCTC 9343 | NCTC 10583 | NCTC 8503 | ATCC 8503 | ATCC 8503 | ATCC 8482 | ATCC 8482 | ATCC 8483 | ATCC 8483 | ATCC 8492 | ATCC 8492 | ATCC 8492 | ATCC 8492 | NCTC 10582 | NCTC 10582 |
| | NCTC 8560 | NCTC 8560 | NCTC 8560 | NCTC 8560 | NCTC 8560 | NCTC 8560 | NCTC 8560 | NCTC 8560 | NCTC 8560 | NCTC 8560 | NCTC 8560 | NCTC 8560 | NCTC 8560 | NCTC 8560 | NCTC 8560 | NCTC 8560 |
| | NCTC 9344 | NCTC 9344 | NCTC 9344 | NCTC 9344 | NCTC 9344 | NCTC 9344 | NCTC 9344 | NCTC 9344 | NCTC 9344 | NCTC 9344 | NCTC 9344 | NCTC 9344 | NCTC 9344 | NCTC 9344 | NCTC 9344 | NCTC 9344 |
| | NCTC 10581 | NCTC 10581 | NCTC 10581 | NCTC 10581 | NCTC 10581 | NCTC 10581 | NCTC 10581 | NCTC 10581 | NCTC 10581 | NCTC 10581 | NCTC 10581 | NCTC 10581 | NCTC 10581 | NCTC 10581 | NCTC 10581 | NCTC 10581 |
| | NCTC 10584 | NCTC 10584 | NCTC 10584 | NCTC 10584 | NCTC 10584 | NCTC 10584 | NCTC 10584 | NCTC 10584 | NCTC 10584 | NCTC 10584 | NCTC 10584 | NCTC 10584 | NCTC 10584 | NCTC 10584 | NCTC 10584 | NCTC 10584 |
| No. of laboratory isolates conforming | 47 | 1 | 4 | 12 | 1 | 4 | 0 | 2 | 1 | 2 | 0 | 1 | 3 | 2 | 7 | 1 |
| Total no. of strains conforming | 52 | 1 | 5 | 12 | 1 | 4 | 1 | 3 | 1 | 2 | 1 | 1 | 3 | 3 | 8 | 1 |

* + = positive result; - = negative result; \perp = weak reaction.

only in the ability to hydrolyse dextran.

B. oralis NP 333 and B. ochraceus VPI 2845 gave individual patterns (Bxix and Bxx) that only differed in the fermentation of rhamnose. The remaining 20 isolates gave different patterns that were intermediate between the patterns obtained with the reference strains.

"M-strains". The results obtained with the 41 strains of B. melaninogenicus are shown in Table 14. Twelve patterns of results were obtained which were characterised by the production of black-pigmented colonies, a zone of complete haemolysis and the digestion of gelatin (except one strain, pattern Mxi, which did not digest gelatin).

Most strains fell into two groups clearly differentiated on the basis of carbohydrate fermentation tests. The two reference strains of B. melaninogenicus ss. intermedius NCTC 9336 and 9338, 10 isolates from the gingival crevice, and two isolates from clinical specimens, gave similar patterns (Miv and Mv) that differed from each other only in the production of lipase. Five strains gave patterns Miii and Mvi that differed from these reference patterns only in the fermentation of lactose.

The second group of B. melaninogenicus were characterised by the absence of saccharolytic activity. Two similar patterns (Mviii and Mix) were composed of the reference strain of B. melaninogenicus ss. asaccharolyticus NCTC 9337, 10 isolates from abdominal wounds and high vaginal swabs, and one oral isolate.

These two patterns differed only in the production of indole. Five asaccharolytic strains failed to grow on supplemented-egg-yolk medium and are therefore placed in a separate pattern (Mxii).

B. melaninogenicus ss. melaninogenicus VPI 4196 and B. melaninogenicus (formerly B. oralis, see Holbrook and Duerden, 1974) ATCC 15930 gave similar patterns characterised by the hydrolysis of dextran and the failure to produce indole; they differed in the production of lipase and in the hydrolysis of aesculin by strain ATCC 15930.

The remaining three strains gave individual patterns of results (Mvii, Mx and Mxi) with those strains in Mx and Mxi being the only Bacteroides strains except for B. corrodens that reduced nitrate to nitrite.

"F-strains". The results of biochemical tests with reference strains of Fusobacterium spp. and a small miscellaneous group of laboratory isolates (F-strains) are given in Table 15. The four referred strains of B. corrodens gave a characteristic pitting or corroding effect when grown on blood agar. This feature was not, however, always observed in older cultures or on subcultures. Commonly a rhizoid or faint spreading growth were more characteristic of these strains after a number of subcultures. These strains were further characterised by the reduction of nitrate and by a marked unreactivity in the tests employed. H_2S was produced and gelatin was digested, but all other tests gave negative results.

TABLE 14

The results of biochemical tests on H-strains

| Test | Pattern of results* | | | | | | | | | | | |
|---|---------------------|----------|------|-----------|-----------|-----|------|-------|-----------|-----|-----|-------|
| | Mi | Mii | Miii | Miv | Mv | Mvi | Mvii | Mviii | Mix | Mx | Mxi | Mxii† |
| H ₂ S production | + | + | + | + | + | + | + | + | + | + | + | + |
| Nitrate reduction | - | - | - | - | - | - | - | - | - | - | - | - |
| Haemolysis on blood agar | + | + | + | + | + | + | + | + | + | + | + | + |
| Lipase production on EYA | + | - | + | + | - | - | - | - | - | - | - | ... |
| Digestion of gelatin | + | + | + | + | + | + | + | + | + | + | + | + |
| Indole production | - | - | + | + | + | + | - | - | + | + | - | + |
| Hydrolysis of aesculin | + | - | - | - | - | - | - | - | - | - | - | - |
| Fermentation of: | | | | | | | | | | | | |
| glucose | + | + | + | + | + | + | + | - | - | - | + | - |
| lactose | + | + | + | - | - | + | - | - | - | - | + | - |
| maltose | + | + | + | + | + | + | + | - | - | - | - | - |
| sucrose | + | + | + | + | + | + | + | - | - | - | + | - |
| rhamnose | - | - | - | - | - | - | - | - | - | - | - | - |
| trehalose | - | - | - | - | - | - | - | - | - | - | - | - |
| mannitol | - | - | - | - | - | - | - | - | - | - | - | - |
| Hydrolysis of dextran | + | + | - | - | - | - | - | - | - | - | - | - |
| Reference strains conforming to the given pattern | ATCC 15930 | VPI 4196 | ... | NCTC 9338 | NCTC 9336 | ... | ... | ... | NCTC 9337 | ... | ... | ... |
| No. of laboratory isolates conforming | 0 | 0 | 1 | 11 | 1 | 4 | 1 | 4 | 7 | 1 | 1 | 5 |
| Total no. of strains conforming | 1 | 1 | 1 | 12 | 2 | 4 | 1 | 4 | 8 | 1 | 1 | 5 |

* + = positive result; - = negative result.

† Five laboratory isolates that did not grow on EYA are shown in the Tables as biochemical group Mxii.

TABLE 15

The results of biochemical tests on *F*-strains

| Test | Pattern of results* | | | | | | | | | |
|---|---------------------|--|------|------------|------------|------------|------|-------|-----------|------------|
| | Fi | Fii | Fiii | Fiv | Fv | Fvi | Fvii | Fviii | Fix | Fx |
| H ₂ S production | + | + | - | + | - | + | - | + | + | - |
| Nitrate reduction | + | - | - | - | - | - | - | - | - | + |
| Haemolysis on blood agar | + | + | + | - | - | - | - | - | - | + |
| Lipase production on EYA | + | + | + | - | - | - | - | - | - | + |
| Digestion of gelatin | + | - | - | - | - | - | - | - | - | - |
| Indole production | - | + | - | + | - | - | - | - | - | - |
| Hydrolysis of aesculin | - | - | - | - | + | + | - | - | + | - |
| Fermentation of | | | | | | | | | | |
| glucose | - | + | + | - | + | - | + | - | + | - |
| lactose | - | + | + | - | + | - | + | - | + | - |
| maltose | - | + | + | - | + | - | + | - | + | - |
| sucrose | - | + | - | - | + | - | + | - | + | - |
| rhamnose | - | - | - | - | - | - | - | - | - | - |
| trehalose | - | - | - | - | + | + | - | - | - | - |
| mannitol | - | - | - | - | - | - | - | - | - | - |
| Hydrolysis of dextran | - | - | - | - | - | - | - | - | - | - |
| Reference strains conforming to the given pattern | ... | NCTC 10575 NCTC 10576 NCTC 10577 | ... | NCTC 10562 | NCTC 10249 | NCTC 10723 | ... | ... | NCTC 7155 | NCTC 10206 |
| No. of laboratory isolates conforming | 4 | 0 | 1 | 1 | 0 | 0 | 1 | 2 | 0 | 0 |
| Total no. of strains conforming | 4 | 3 | 1 | 2 | 1 | 1 | 1 | 2 | 1 | 1 |

* + = positive result; - = negative result; + = weak reaction.

Three reference strains of F. necrophorum (NCTC 10575, 10576 and 10577) gave almost identical patterns (Fii). They gave variable results in the carbohydrate fermentation tests but the results were never more than weakly positive (a fall in pH of 0.5 unit). These strains were characterised by translucent colonies with a wide zone of complete haemolysis on blood agar; they produced lipase and indole but had little fermentative ability.

F. polymorphum NCTC 10562, F. necrogenes NCTC 10723 and L. buccalis NCTC 10249 gave distinct and individual patterns of results. L. buccalis NCTC 10249 was distinguished by its saccharolytic activity. One clinical and one oral isolate produced only H_2S and were otherwise unreactive, (pattern Fviii), and one clinical isolate gave only weakly positive results in the fermentation tests (Fvii).

The true identity of B. necrophorus (sic) NCTC 7155 is debated; some workers regard it as a Clostridium sp. (Catalogue of the National Collection of Type Cultures, 1972; Barnes and Goldberg, 1968). In these tests the organism was consistently a Gram-negative non-sporing anaerobic bacillus and gave a pattern of results (Fix) that was identical with B. fragilis ss fragilis.

The pattern (Fx) obtained with L. dentium NCTC 10206 is included in this table although this organism does not conform with the general characteristics of the family Bacteroidaceae and the genus Leptotrichia; it is a Gram-positive aerobic organism probably Bacterionema matruchotii (see Gilmour, Howell and Bibby, 1961).

Results of tolerance tests

Sixteen patterns (A-R) of results were obtained with the 165 strains used in these tests of tolerance to a series of dyes and bile salts (Table 16). All the reference strains of B. fragilis ss. fragilis NCTC 8560, 9343, 9344, 10581 and 10584, B. fragilis ss. thetaitaomicron NCTC 10582 and ATCC 8492, B. fragilis ss. ovatus ATCC 8483 and 48 laboratory isolates gave identical patterns (A). They were inhibited by deoxycholate but not by a mixture of deoxycholate and taurocholate and grew in the presence of Victoria blue 4R. Fifteen strains of clinical isolates gave a similar pattern (B) in which the inhibition of growth by deoxycholate was not overcome by the presence of taurocholate. B. fragilis ss. vulgatus NCTC 10583 and 15 laboratory isolates gave a pattern (E) characterised by growth in the presence of Victoria blue 4R and ethyl violet. The other reference strain of B. fragilis ss. vulgatus ATCC 8482 was inhibited by these dyes and gave the same pattern (D) as B. fragilis ss. distasonis ATCC 8503.

All reference strains of B. melaninogenicus, NCTC 9336, 9337, 9338, ATCC 15930 and VPI 4196, as well as 30 laboratory isolates of B. melaninogenicus, gave an identical tolerance pattern (K); growth was inhibited by both bile salts and all four dyes. B. ochraceus VPI 2845, L. dentium NCTC 10206, seven laboratory isolates of B. fragilis-like strains and one F-strain gave the same pattern (K). B. oralis NP 333, two laboratory isolates of B. melaninogenicus, two F-strains, and three B. fragilis-like strains gave the same pattern (J) that differed from the main

TABLE 16

The results of tolerance tests with 165 strains of
Gram-negative anaerobic bacilli

| Test | Pattern of results* | | | | | | | | | | | | | | | Q (R)† |
|---|---------------------|-----|-----|-----------|------------|-----|-----|-----|--------|------------|-----|------------|------------|------------|-----------|--------|
| | A | B | C | D | E | F | G | H | J | K | L | M | N | P | Q | |
| Growth on basal medium | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | ... |
| Growth on basal medium plus: | | | | | | | | | | | | | | | | |
| taurocholate (0.5%) | + | + | + | + | + | + | + | + | I | I | I | I | + | I | + | ... |
| deoxycholate (0.1%) | I | I | I | I | I | I | I | + | I | I | I | I | + | I | + | ... |
| taurocholate (0.5%) & deoxycholate (0.1%) | + | I | + | + | + | I | I | + | I | I | I | I | + | I | + | ... |
| Victoria blue 4R | + | + | + | I | + | + | I | + | + | I | + | + | + | + | + | ... |
| (1/80 000) | | | | | | | | | | | | | | | | |
| ethyl violet | I | I | I | I | + | + | I | I | I | I | I | + | + | + | + | ... |
| (1/80 000) | | | | | | | | | | | | | | | | |
| gentian violet | I | I | I | I | I | I | I | I | I | I | I | + | + | + | + | ... |
| (1/100 000) | | | | | | | | | | | | | | | | |
| brilliant green | I | I | + | I | I | I | I | I | I | I | + | + | I | I | + | ... |
| (1/80 000) | | | | | | | | | | | | | | | | |
| Reference strains conforming to the given pattern | NCTC 8560 | ... | ... | ATCC 8482 | NCTC 10583 | ... | ... | ... | NP 333 | NCTC 9336 | ... | NCTC 10575 | NCTC 10723 | NCTC 10249 | NCTC 7155 | ... |
| | NCTC 9343 | | | ATCC 8503 | | | | | | NCTC 9337 | | NCTC 10576 | | | | |
| | NCTC 9344 | | | | | | | | | NCTC 9338 | | NCTC 10577 | | | | |
| | NCTC 10581 | | | | | | | | | VPI 4196 | | NCTC 10562 | | | | |
| | NCTC 10582 | | | | | | | | | VPI 2845 | | | | | | |
| | NCTC 10584 | | | | | | | | | ATCC | | | | | | |
| | ATCC 8483 | | | | | | | | | NCTC 15930 | | | | | | |
| | ATCC 8492 | | | | | | | | | 10206 | | | | | | |
| No. of laboratory isolates conforming | 48 | 15 | 2 | 0 | 15 | 1 | 1 | 1 | 7 | 38 | 6 | 2 | 0 | 0 | 0 | 3 |
| Total no. of strains conforming | 56 | 15 | 2 | 2 | 16 | 1 | 1 | 1 | 8 | 45 | 6 | 6 | 1 | 1 | 1 | 3 |

* + = growth; I = inhibition of growth.

† Three laboratory isolates of *B. melaninogenicus* that did not grow on tolerance test basal medium are shown in the tables as tolerance group R.

B. melaninogenicus pattern by growth in the presence of Victoria blue 4R.

Results of antibiotic-disk-resistance tests

The patterns of results obtained with the 165 test strains in antibiotic-disk-resistance tests are shown in Tables 17a and 17b. The 105 B. fragilis-like strains gave 18 patterns (1-18); 61 strains gave a single pattern (1) characterised by resistance to neomycin, kanamycin, penicillin, methicillin, colistin, the 2 µg lincomycin disk, bacitracin and vancomycin. The remaining 44 strains differed from this typical pattern to a greater or lesser extent. All strains were resistant to the 10 µg neomycin and 30 µg kanamycin disks and only one strain (pattern 17) was sensitive to the 1000 µg kanamycin disk; but 15 strains (patterns 12-18) were sensitive to the 1000 µg neomycin disk; all except two strains (patterns 4 and 17) were resistant to vancomycin. Two strains were sensitive to colistin (pattern 18) and three strains were sensitive to bacitracin (patterns 15 and 17). B. fragilis-like strains were typically resistant to penicillin but 6 strains were sensitive to both penicillin and methicillin (patterns 10 and 11) and a further two strains to methicillin alone (pattern 18). Only three strains, including B. fragilis ss. thetataomicron ATCC 8492 and B. fragilis ss. ovatus ATCC 8483 were resistant to rifampicin (patterns 6, 7 and 8). B. fragilis ss. ovatus ATCC 8483 and B. fragilis ss. distasonis ATCC 8503 were the only strains resistant to chloramphenicol (patterns 7 and 16). Fourteen strains including B. fragilis ss. fragilis NCTC 8560

TABLE 17a

The results of antibiotic-disk-resistance tests with 165 strains of
Cran-negative anaerobic bacilli

| Antibiotic (content per disc) | Patterns of results* | | | | | | | | | | | | | | | | | | | |
|---|--|--------------|---------------|---------------|-----|-----------|-----------|-----|-----|-----|-----|-----|-----------|-----|-----|-----------|-----|-----|-----|-----|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 |
| Neomycin (1000 µg) | R | R | R | R | R | R | R | R | R | R | R | S | S | S | S | S | S | S | R | R |
| Neomycin (10 µg) | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R |
| Kanamycin (1000 µg) | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R |
| Kanamycin (30 µg) | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R |
| Penicillin (1.5 units) | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R |
| Methicillin (10 µg) | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R |
| Erythromycin (60 µg) | S | R | S | S | S | S | R | R | R | S | S | R | S | S | S | S | S | S | S | S |
| Colistin (10 µg) | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R |
| Rifampicin (15 µg) | S | S | S | S | S | S | R | R | S | S | S | S | S | S | S | S | S | S | S | S |
| Lincomycin (2 µg) | S | S | R | S | S | S | S | R | S | S | S | R | R | S | S | S | S | S | S | S |
| Clindamycin (2 µg) | S | S | R | S | S | S | S | R | S | S | S | S | S | S | S | S | S | S | S | S |
| Bacitracin (0.1 unit) | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R |
| Vancomycin (15 µg) | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R |
| Chloramphenicol (10 µg) | S | S | S | S | S | S | R | S | S | S | S | S | S | S | S | R | S | S | S | S |
| Reference strains conforming to the pattern | NCTC 9343 NCTC 9344 NCTC 10583 NCTC 10584 | NCTC 8560 | NCTC 10582 | NCTC 10581 | ... | ATCC 8492 | ATCC 8483 | ... | ... | ... | ... | ... | ATCC 8482 | ... | ... | ATCC 8503 | ... | ... | ... | ... |
| No. of laboratory isolates conforming | 57 | 7 | 4 | 0 | 5 | 0 | 0 | 1 | 1 | 5 | 1 | 3 | 3 | 2 | 2 | 0 | 1 | 2 | 2 | 1 |
| Total no. of strains conforming | 61 | 8 | 5 | 1 | 5 | 1 | 1 | 1 | 1 | 5 | 1 | 3 | 4 | 2 | 2 | 1 | 1 | 2 | 2 | 1 |

* S = sensitive; R = resistant

TABLE 17b

The results of antibiotic-disk-resistance tests with 165 strains of
Gram-negative anaerobic bacilli (cont.)

| Antibiotic (content per disc) | Patterns of results* | | | | | | | | | | | | | | | | | | | | |
|---|----------------------|-----|-----|-----------|-----|-----------|------------|----------|-----|-----|----------|-----|-----|------------|-----|------------|------------|-----------|-----|-----|------------|
| | 21 | 22 | 23 | 24 | 25 | 26 | 27 | 28 | 29 | 30 | 31 | 32 | 33 | 34 | 35 | 36 | 37 | 38 | 39 | 40 | 41 |
| Neomycin (1000 µg) | R | R | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S |
| Neomycin (10 µg) | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R |
| Kanamycin (1000 µg) | R | R | R | R | R | R | R | R | R | S | S | R | R | S | S | S | S | S | S | S | S |
| Kanamycin (30 µg) | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | S | R | R | S | S | S |
| Penicillin (1.5 units) | S | S | R | S | S | S | S | S | S | R | S | R | R | S | S | S | S | S | S | S | S |
| Methicillin (10 µg) | S | S | R | S | S | S | S | S | S | R | S | S | R | S | S | S | S | S | R | S | S |
| Erythromycin (60 µg) | S | S | S | S | S | S | S | S | S | S | S | S | S | S | R | S | S | R | S | S | S |
| Colistin (10 µg) | S | R | S | S | S | R | R | S | S | S | R | R | R | S | S | S | S | S | S | S | R |
| Rifampicin (15 µg) | S | S | S | S | S | S | S | S | S | S | S | S | S | R | S | S | S | R | R | S | S |
| Lincomycin (2 µg) | S | S | S | S | S | S | S | S | S | R | S | S | S | S | S | S | S | S | S | S | S |
| Clindamycin (2 µg) | S | S | S | S | S | S | S | S | S | R | R | S | S | S | S | S | S | S | S | S | S |
| Bacitracin (0.1 unit) | S | R | S | S | S | S | R | R | S | R | R | R | R | R | R | S | R | R | R | S | S |
| Vancomycin (15 µg) | S | S | R | R | S | S | S | R | R | S | S | R | S | R | R | S | R | R | S | S | S |
| Chloramphenicol (10 µg) | R | S | S | S | S | S | S | S | R | S | S | S | S | S | S | S | S | S | S | S | S |
| Reference strains conforming to the pattern | ... | ... | ... | NCTC 9336 | ... | NCTC 9337 | ATCC 15930 | VPI 4196 | ... | ... | VPI 2845 | ... | ... | NCTC 10575 | ... | NCTC 10249 | NCTC 10562 | NCTC 7155 | ... | ... | NCTC 10206 |
| | | | | NCTC 9338 | | | | NP 333 | | | | | | NCTC 10576 | | | | | | | |
| | | | | | | | | | | | | | | NCTC 10577 | | | | | | | |
| | | | | | | | | | | | | | | NCTC 10723 | | | | | | | |
| No. of laboratory isolates conforming | 1 | 1 | 2 | 15 | 3 | 4 | 2 | 2 | 2 | 1 | 0 | 1 | 1 | 0 | 1 | 1 | 0 | 0 | 1 | 4 | 0 |
| Total no. of strains conforming | 1 | 1 | 2 | 17 | 3 | 5 | 3 | 4 | 2 | 1 | 1 | 1 | 1 | 4 | 1 | 2 | 1 | 1 | 1 | 4 | 1 |

* S = sensitive; R = resistant

and B. fragilis ss. ovatus ATCC 8483 were resistant to erythromycin (patterns 2, 7, 8, 9 and 12). Twenty-one strains including B. fragilis ss. fragilis NCTC 10581, B. fragilis ss. thetaitaomicron ATCC 8492 and B. fragilis ss. distasonis ATCC 8503 were sensitive to the 2 µg lincomycin disk (patterns 4, 5, 6, 9, 10, 14, 15, 16, 17 and 18). All except six strains (patterns 3 and 8), however, were sensitive to the clindamycin disk. One reference strain, B. fragilis ss. thetaitaomicron NCTC 10582, was resistant to clindamycin.

The 41 B. melaninogenicus strains gave 12 patterns of results (19-30). Seventeen strains, including B. melaninogenicus ss. intermedius NCTC 9336 and 9338, gave a single pattern (24) characterised by resistance to the 10 µg neomycin disk, 30 µg and 1000 µg kanamycin disks and vancomycin, and sensitivity to the other antibiotics. All B. melaninogenicus strains were sensitive to erythromycin and rifampicin. Three strains were resistant to penicillin and methicillin (patterns 23 and 30), three to chloramphenicol (patterns 21 and 29), and one strain (pattern 30) was resistant to lincomycin and clindamycin. Five strains (patterns 19-22) were resistant to the 1000 µg neomycin disk, three were sensitive to the 1000 µg kanamycin disk (patterns 29 and 30), and 15 strains, including B. melaninogenicus ss. asaccharolyticus NCTC 9337 and B. melaninogenicus ATCC 15930 were sensitive to vancomycin (patterns 20-22, 25-27 and 30). Twelve strains, including B. melaninogenicus ss. asaccharolyticus NCTC 9337 and B. melaninogenicus ATCC 15930 were resistant to colistin (patterns 19, 20, 22, 26 and 27) and eight strains including B. melaninogenicus

ATCC 15930 and B. melaninogenicus ss. melaninogenicus VPI 4196 were resistant to bacitracin (patterns 22, 27, 28 and 30).

B. oralis NP 333 and B. ochraceus VPI 2845 gave similar patterns (28 and 31) to the B. melaninogenicus strains. B. oralis NP 333 gave the same pattern (28) as B. melaninogenicus ss. melaninogenicus VPI 4196 and two laboratory isolates of B. melaninogenicus.

B. ochraceus VPI 2845 differed from the typical B. melaninogenicus pattern in sensitivity to the 1000 µg kanamycin disk and vancomycin, and resistance to colistin, clindamycin and bacitracin.

The 17 F-strains gave 10 patterns of results (32-41). L. dentium NCTC 10206 was sensitive to all disks except colistin (pattern 41). All four strains of B. corrodens gave the same pattern (40); they were sensitive to all disks except rifampicin and bacitracin. The remaining F-strains were sensitive to the 1000 µg neomycin disk, lincomycin, clindamycin and chloramphenicol and only two strains (patterns 32 and 33) were resistant to the 1000 µg kanamycin disk, colistin and penicillin. All except one strain (pattern 39) were resistant to the 10 µg neomycin disk; three strains were sensitive to the 30 µg kanamycin disk (patterns 36 and 39) and four strains (patterns 33, 36 and 39) were sensitive to the vancomycin disk. Only two strains (pattern 36) were sensitive to bacitracin. Two strains were resistant to methicillin (patterns 33 and 39) and three were resistant to erythromycin (patterns 35, 38 and 39). F. necrophorum NCTC 10575, 10576 and 10577 and F. necrogenes NCTC 10723 were resistant to rifampicin and gave identical patterns (34). B. necrophorus NCTC 7155 (pattern 38)

and one laboratory isolate (pattern 39) were also resistant to rifampicin.

With two exceptions, all strains were found to be sensitive to metronidazole. The exceptions were L. dentium NCTC 10206 and B. ochraceus VPI 2845 which was only tested after it had been noted that this organism was growing in air with 10% CO₂. The zone diameter was usually graded ++ (26-35 mm) for B. fragilis strains whereas B. melaninogenicus and Fusobacterium strains produced zone diameters considerably in excess of 36 mm. An exception to this general finding was a small number of B. melaninogenicus ss. melaninogenicus strains which had a small zone around this disk, usually graded ++. A frequent observation was the elliptical shape of the zone of inhibition and diameters were recorded as an average of two diameters.

Analysis of results

After the Tables 13-17a and b inclusive had been drawn up, a comparison of the results obtained in the biochemical tests (Tables 13, 14 and 15) was made with those results obtained in the tolerance tests (Table 28). This comparison is shown in Tables 18, 19 and 20. A further comparison was made using the results obtained in biochemical tests and those for the antibiotic-disk-resistance tests and this is shown in Tables 21, 22 and 23. The number of strains which have the patterns of results indicated by the co-ordinates of Tables 18-23 inclusive is shown and the

position of reference strains in these tables is indicated by a superscript.

Comparison of results of biochemical tests with the results of tolerance tests

Forty-nine of the 52 strains that gave the biochemical pattern Bi (B. fragilis ss. fragilis) and 19 of the 22 strains that gave patterns Bviii-Bxv and contained all B. fragilis ss. ovatus and B. fragilis ss. thetaitaomicron strains gave two patterns of tolerance, A and B. The 15 strains in tolerance pattern B differed from the more common pattern A only in their inability to grow in the deoxycholate and taurocholate mixture. The 17 strains of B. fragilis ss. vulgatus in biochemical groups Biii and Biv gave distinct patterns of tolerance, E and F, distinguished by growth in the presence of ethyl violet and the one strain in pattern F differed from pattern E only in its ability to grow in the presence of the deoxycholate and taurocholate mixture. Eleven strains that gave biochemical patterns conforming with the B. fragilis-like group (patterns Bi-Bxviii) gave patterns of tolerance that were the same as patterns obtained with B. melaninogenicus strains (patterns J, K and L). These strains were all inhibited by taurocholate, and seven of them gave biochemical patterns that did not conform with the typical B. fragilis sub-species patterns.

B. oralis NP 333 and B. ochraceus VPI 2845 (biochemical patterns Bxix and Bxx) also gave tolerance patterns J and K.

Thirty-five of the 41 strains of B. melaninogenicus

TABLE 18

A comparison of the results obtained in the biochemical tests with those obtained in the tolerance tests for the B-strains

| Biochemical pattern | Number of strains with the stated tolerance pattern | | | | | | | | | | | | | | | | Key to position of reference strain(s) (indicated by superscript) |
|---------------------|---|---|---|----------------|----------------|---|---|---|----------------|---|----------------|---|---|---|---|---|---|
| | A | B | C | D | E | F | G | H | I | J | K | L | M | N | P | Q | |
| Bi | 41 ^a | 8 | 2 | | | | | | 1 | | | | | | | | a <i>B. fragilis</i> ss. <i>fragilis</i> NCTC 9343, 9344, 10581, 10584 and 8560 |
| Bii | | | | | | | | | | 1 | | | | | | | b <i>B. fragilis</i> ss. <i>vulgatus</i> NCTC 10583 |
| Biii | | | | | 4 ^b | 1 | | | | | | | | | | | |
| Biv | | | | | 12 | | | | | | | | | | | | |
| Bv | | | | | | | | | | 1 | | | | | | | |
| Bvi | | | | | | | | | 1 | 2 | 1 | | | | | | |
| Bvii | | | | 1 ^c | | | | | | | | | | | | | c <i>B. fragilis</i> ss. <i>distasonis</i> ATCC 8503 |
| Bviii | 2 | | | 1 ^d | | | | | | | | | | | | | d <i>B. fragilis</i> ss. <i>vulgatus</i> ATCC 8482 |
| Bix | | | 1 | | | | | | | | | | | | | | |
| Bx | | | 2 | | | | | | | | | | | | | | |
| Bxi | 1 ^e | | | | | | | | | | | | | | | | e <i>B. fragilis</i> ss. <i>ovatus</i> ATCC 8483 |
| Bxii | | 1 | | | | | | | | | | | | | | | |
| Bxiii | 2 | | | | | | | | | 1 | | | | | | | f <i>B. fragilis</i> ss. <i>thetaiotaomicron</i> ATCC 8492 |
| Bxiv | 3 ^f | | | | | | | | | | | | | | | | g <i>B. fragilis</i> ss. <i>thetaiotaomicron</i> NCTC 10582 |
| Bxv | 7 ^g | | | | | 1 | | | | | | | | | | | |
| Bxvi | | | | | | | | 1 | | | | | | | | | |
| Bxvii | 2 | 1 | | | | | | | | | 2 | | | | | | h <i>B. oralis</i> NP 333 |
| Bxviii | | | | | | | | | | | 1 | | | | | | j <i>B. ochraceus</i> VPI 2845 |
| Bxix | | | | | | | | | 1 ^h | | | | | | | | |
| Bxx | | | | | | | | | | | 1 ^j | | | | | | |

TABLE 20

A comparison of the results obtained in the biochemical tests with those obtained in the tolerance tests for the F-strains

| Biochemical pattern | Number of strains with the stated tolerance pattern | | | | | | | | | | | | | | | Key to position of reference strain(s) (indicated by superscript) |
|---------------------|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | A | B | C | D | E | F | G | H | J | K | L | M | N | P | Q | |
| Fi | | | | | | | | | | | | | | | | q <i>F. necrophorum</i> NCTC 10575, 10576 and 10577 |
| Fii | | | | | | | | | | | | | | | | |
| Fiii | | | | | | | | | | | | | | | | |
| Fiv | | | | | | | | | | | | | | | | r <i>F. polymorphum</i> NCTC 10562 |
| Fv | | | | | | | | | | | | | | | | s <i>L. buccalis</i> NCTC 10249 |
| Fvi | | | | | | | | | | | | | | | | t <i>F. necrogenes</i> NCTC 10723 |
| Fvii | | | | | | | | | | | | | | | | |
| Fviii | | | | | | | | | | | | | | | | |
| Fix | | | | | | | | | | | | | | | | u <i>B. necrophorus</i> NCTC 7155 |
| Fx | | | | | | | | | | | | | | | | v <i>L. dentium</i> NCTC 10206 |

(biochemical patterns Mi-Mxii) gave a single tolerance pattern, K. Three strains grew in the presence of Victoria blue 4R and gave tolerance patterns J and L and the remaining three strains failed to grow on the tolerance-test basal medium.

The four strains of B. corrodens that gave tolerance pattern L all gave biochemical pattern Fi. F. necrophorum NCTC 10575, 10576 and 10577 (biochemical pattern Fii), F. polymorphum NCTC 10562 (biochemical pattern Fiv) and two laboratory isolates (biochemical patterns Fiv and Fviii) gave tolerance pattern M distinguished by growth in the presence of all dyes and inhibition of growth by bile salts. F. necrogenes NCTC 10723 (biochemical pattern Fvi) gave tolerance pattern N; it grew in the presence of all the bile salts and dyes except brilliant green.

L. buccalis NCTC 10249 (biochemical pattern Fv) gave tolerance pattern P; it grew in all dyes except brilliant green but was inhibited by the bile salts. Four F-strains, including L. dentium NCTC 10206 (biochemical pattern Fx) gave tolerance patterns J and K. B. necrophorus NCTC 7155 (biochemical pattern Fix) was the only test strain which grew in the presence of all the bile salts and dyes used (tolerance pattern Q).

Comparison of the results of the biochemical tests with those of the antibiotic-disk-resistance tests

Sixty-one of the 105 B. fragilis-like strains (biochemical patterns Bi-Bxviii) gave a single pattern of antibiotic-resistance (pattern 1) and eight differed only in resistance to erythromycin

(pattern 2). There were no differences between the recognised sub-species of B. fragilis. The strains that differed from these typical patterns of antibiotic resistance, including 15 strains that were sensitive to the 1000 µg neomycin disk, were distributed throughout the 18 biochemical patterns.

B. ochraceus VPI 2845 (biochemical pattern Bxx) gave a unique pattern of resistance (pattern 31) and B. oralis NP333 (biochemical pattern Bxix) gave the same pattern as three B. melaninogenicus strains (pattern 28). The 41 strains of B. melaninogenicus (biochemical patterns Mi-Mxii) gave 12 patterns of resistance to antibiotics (patterns 19-30). Sixteen saccharolytic strains of B. melaninogenicus and one asaccharolytic strain gave pattern 24. The distribution of the remaining 24 strains between the other 11 resistance patterns was unrelated to the biochemical patterns.

All four strains of B. corrodens gave unique biochemical (Fi) and antibiotic resistance (40) patterns. The three patterns of F. necrophorum NCTC 10575, 10576 and 10577 (biochemical pattern Fii) and F. necrogenes NCTC 10723 (biochemical pattern Fvi) gave the same antibiotic resistance pattern (34). The remaining F-strains gave varied antibiotic resistance patterns.

There was no overlap between the patterns of antibiotic resistance obtained with B. fragilis-like strains (biochemical patterns Bi-Bxviii), B. melaninogenicus strains (patterns Mi-Mxii) and F-strains (Fi-Fx). B. oralis NP333 and B. ochraceus VPI 2845

TABLE 21

A comparison of the results obtained in the biochemical tests with those for the antibiotic-disk-resistance tests for the B-strains

| Bio-chemical pattern | Number of strains with the stated pattern of antibiotic resistance | | | | | | | | | | | | | | | | | | | | | Key to position of reference strain(s) (indicated by superscript) |
|----------------------|--|----------------|----------------|----------------|---|----------------|---|---|---|----|----|----|----|----|----------------|----------------|----|----------------|----|----------------|----|--|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 28 | 31 | 41 | |
| Bi | 36 ^a | 8 ^b | | 1 ^c | 2 | | 1 | 1 | | | 3 | | | | | | | | | | | |
| Bii | | | | | | | | | | | | | | | | | 1 | | | | | |
| Biii | | 2 ^d | | 2 | | | | | | | 1 | | | | | | | | | | | |
| Biv | 11 | | | 1 | | | | | | | | | | | | | | | | | | |
| Bv | | | | | | | | | | | | | | | | | 1 | | | | | |
| Bvi | | | | | | | | | | | | | | | | | | | | | | |
| Bvii | | | | | | | | | | | | | | | | | | | | | | |
| Bviii | 2 | | | | | | | | | | | | | | | 1 ^e | | | | | | |
| Bix | 1 | | | | | | | | | | | | | | 1 ^f | | | | | | | |
| Bx | | | | | | | | | | | | | | | | | | | | | | |
| Bxi | | | | | | | | | | | | | | | | | | | | | | |
| Bxii | | | | | | | | | | | | | | | | | | | | | | |
| Bxiii | | | | | | | | | | | | | | | | | | | | | | |
| Bxiv | 1 | | | 1 | | | | | | | | | | | | | | | | | | |
| Bxv | 4 | | 3 ^j | | | 1 ^h | | | | | | | | | | | | | | | | |
| Bxvi | 1 | | | | | | | | | | | | | | | | | | | | | |
| Bxvii | 3 | | | | | | | | | | | | | | | | | | | | | |
| Bxviii | | | | | | | | | | | | | | | | | | | | | | |
| Bxix | | | | | | | | | | | | | | | | | | | | | | |
| Bxx | | | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | 1 ^k | | 1 ^l | | |
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a *B. fragilis* ss. *fragilis* NCTC 9343, 9344 and 10584b *B. fragilis* ss. *fragilis* NCTC 8560c *B. fragilis* ss. *fragilis* NCTC 10581d *B. fragilis* ss. *vulgatus* NCTC 10583e *B. fragilis* ss. *distasonis* ATCC 8503f *B. fragilis* ss. *vulgatus* ATCC 8482g *B. fragilis* ss. *ovatus* ATCC 8483h *B. fragilis* ss. *thetaiotaomicron* ATCC 8492j *B. fragilis* ss. *thetaiotaomicron* NCTC 10582k *B. oralis* NP 333l *B. ochraceus* VPI 2845

(biochemical patterns Bxix and Bxx respectively) gave patterns similar to B. melaninogenicus strains.

Results of tests on strains listed in Appendix Ib

The 53 strains listed in Appendix Ib were collected after the original identification scheme had been drawn up. These strains were examined by the full series of tests as described for the strains listed in Appendix Ia. The patterns of results obtained with these strains were compared with those patterns obtained for the strains listed in Appendix Ia. Identification of the strains was made where possible and it was noted whether or not the patterns obtained with each of the test strains were similar to one of the series of patterns obtained with the strains listed in Appendix Ia. This is indicated in Table 24.

From the patterns of results obtained with the 218 strains listed in Appendices Ia and Ib a number of tests were noted to be of particular value in discriminating between the reference strains and in placing most of the test strains into clearly defined groups. This shortened series of tests is given in Table 5. The reactions of the 53 strains in Appendix Ib to this series of tests was examined. The ability of each of these organisms to fit into a recognised pattern in this identification scheme is also noted in Table 24. The identification scheme is further discussed on page 150, and the scheme itself is given as fig. (v).

† Pigment production (black colonies) observed on lysed blood agar after anaerobic incubation for up to 7 days.

TABLE 24Identification of the 53 strains listed in Appendix Ib

| Strain number | Strains fitting a pattern in the full series of tests | Strains fitting a pattern in the shortened series of tests (Table 5) | Identification |
|-------------------------------------|--|--|--|
| NCTC 10560 | - | - | <u>Sphaerophorus</u> <u>varians</u> |
| <u>B. corrodens</u> 151/RV | - | + | <u>B. corrodens</u> |
| " RV/2 | - | + | <u>B. corrodens</u> |
| " 143A | - | + | <u>B. corrodens</u> |
| <u>B. oralis</u> J1 | - | - | <u>B. oralis</u> ¹ |
| " 7CM | - | - | <u>B. oralis</u> ¹ |
| " 30 ² | + | + | <u>B. melanino-</u> <u>genicus</u> ss. <u>melaninogenicus</u> |
| <u>B. melaninogenicus</u> G11a-d | + | + | <u>B. melanino-</u> <u>genicus</u> ss. <u>intermedius</u> |
| " P11a-k | + | + | <u>B. melanino-</u> <u>genicus</u> ss. <u>intermedius</u> |
| " AB13a-f | + | + | <u>B. melanino-</u> <u>genicus</u> ss. <u>asaccharolyticus</u> |
| " UJB13a-c | + | + | <u>B. melanino-</u> <u>genicus</u> ss. <u>intermedius</u> |
| " D13a-f | + | + | <u>B. melanino-</u> <u>genicus</u> ss. <u>intermedius</u> |

TABLE 24 (continued)

| | | | |
|---|---|---|--|
| <u>Bacteroides</u> sp | | | |
| B20-a ³ | - | - | aerobe (? <u>Eikenella</u> sp) |
| <u>Laboratory isolates</u> | | | |
| GNAB 45 | - | - | unknown |
| GNAB 46 | - | - | unknown |
| GNAB 47 | - | - | unknown |
| WPH 94 | + | + | <u>B. melanino-</u> <u>genicus</u> ss. <u>asaccharolyticus</u> |
| WPH 95 | + | + | <u>B. melanino-</u> <u>genicus</u> ss. <u>asaccharolyticus</u> |
| WPH 96 | - | - | ? <u>B. corrodens</u> |
| WPH 110 | - | - | ? <u>B. melanino-</u> <u>genicus</u> ss. <u>asaccharolyticus</u> |
| WPH 111 | + | + | <u>B. melanino-</u> <u>genicus</u> ss. <u>asaccharolyticus</u> |
| WPH 112 | + | + | <u>B. melanino-</u> <u>genicus</u> ss. <u>asaccharolyticus</u> |
| WPH 113 | + | + | <u>B. melanino-</u> <u>genicus</u> ss. <u>asaccharolyticus</u> |
| WPH 114 | - | + | <u>B. melanino-</u> <u>genicus</u> ss. <u>asaccharolyticus</u> |
| <u>Gingival crevice</u> <u>strains</u> | | | |
| WPH 60 | + | + | <u>B. melanino-</u> <u>genicus</u> ss. <u>intermedius</u> |

TABLE 24 (continued)

| | | | |
|--------|---|---|---|
| WPH 61 | + | + | <u>B. oralis</u> |
| WPH 62 | + | + | <u>B. melanino-</u> <u>genicus ss.</u> <u>melaninogenicus</u> |
| WPH 63 | + | + | <u>B. melanino-</u> <u>genicus ss.</u> <u>melaninogenicus</u> |
| WPH 64 | + | + | <u>B. melanino-</u> <u>genicus ss.</u> <u>melaninogenicus</u> |
| WPH 65 | - | - | ? <u>B. melanino-</u> <u>genicus ss.</u> <u>intermedius</u> |
| WPH 66 | - | + | <u>B. melanino-</u> <u>genicus ss.</u> <u>melaninogenicus</u> |
| WPH 67 | - | + | <u>B. melanino-</u> <u>genicus ss.</u> <u>melaninogenicus</u> |
| WPH 68 | - | + | <u>B. melanino-</u> <u>genicus ss.</u> <u>melaninogenicus</u> |
| WPH 70 | + | + | <u>B. melanino-</u> <u>genicus ss.</u> <u>melaninogenicus</u> |
| WPH 71 | - | + | <u>Fusobacterium sp</u> |
| WPH 72 | - | + | <u>B. melanino-</u> <u>genicus ss.</u> <u>intermedius</u> |
| WPH 73 | + | + | <u>B. melanino-</u> <u>genicus ss.</u> <u>intermedius</u> |
| WPH 74 | + | + | <u>B. melanino-</u> <u>genicus ss.</u> <u>intermedius</u> |

TABLE 24 (continued)

| | | | |
|---------------------|---|---|--|
| WPH 75 | - | + | <u>B. melanino-</u> <u>genicus ss.</u> <u>intermedius</u> |
| WPH 76 | + | + | <u>B. melanino-</u> <u>genicus ss.</u> <u>intermedius</u> |
| WPH 77 | + | + | <u>B. melanino-</u> <u>genicus ss.</u> <u>intermedius</u> |
| WPH 78 | - | + | <u>B. melanino-</u> <u>genicus ss.</u> <u>intermedius</u> |
| WPH 79 | - | + | <u>B. melanino-</u> <u>genicus ss.</u> <u>asaccharolyticus</u> |
| WPH 80 ⁴ | - | - | ? <u>B. melanino-</u> <u>genicus ss.</u> <u>intermedius</u> |
| WPH 81 | + | + | <u>B. melanino-</u> <u>genicus ss.</u> <u>intermedius</u> |
| WPH 82 | + | + | <u>B. melanino-</u> <u>genicus ss.</u> <u>intermedius</u> |
| WPH 83 | + | + | <u>Fusobacterium</u> sp |
| WPH 84 | + | + | <u>B. melanino-</u> <u>genicus ss.</u> <u>intermedius</u> |
| WPH 85 | + | + | <u>B. melanino-</u> <u>genicus ss.</u> <u>intermedius</u> |
| WPH 86 | + | + | <u>B. melanino-</u> <u>genicus ss.</u> <u>asaccharolyticus</u> |

TABLE 24 (continued)

| | | | |
|--------|---|---|---|
| WPH 87 | + | + | <u>B. melanino-</u> <u>genicus ss.</u> <u>intermedius</u> |
| WPH 88 | - | + | <u>B. melanino-</u> <u>genicus ss.</u> <u>melaninogenicus</u> |
| WPH 99 | + | + | <u>B. melanino-</u> <u>genicus ss.</u> <u>intermedius</u> |

Notes relating to Table 24

- + = strain fitting a pattern.
- = strain not fitting a pattern.
- 1 = these two strains of B. oralis were similar to but not identical with each other or to other strains of B. oralis examined.
- 2 = this strain, labelled B. oralis, was found to produce black pigment when grown on blood agar and was identified as B. melaninogenicus ss. melaninogenicus (also see Holbrook and Duerden, 1974).
- 3 = this strain grew aerobically and was provisionally identified as Eikenella corrodens on the basis of its marked pitting of the surface of blood agar plates.
- 4 = this strain did not grow well on solid, blood-free media and was thus impossible to identify with certainty.

Results of tests with the strains listed in Appendix Ic

The strains listed in Appendix Ic were identified using the shortened series of tests listed in Tables 25 and 26.

B. melaninogenicus VPI 9169 was identified as B. melaninogenicus ss. intermedius. Three of the four clinical isolates were B. melaninogenicus but one grew poorly and could not be identified further. The two other isolates of B. melaninogenicus were both identified as subspecies asaccharolyticus and both were resistant to penicillin. One of these two strains was isolated from a dental abscess. The remaining clinical isolate grew poorly and could not be identified.

Thirty-seven of the remaining strains were from the clinical sampling survey (Method 1) and 30 from the survey (Method 2, see pages 67-73). The 37 strains isolated by Method 1 were all B. melaninogenicus and the distribution of these strains among the recognised subspecies is noted in Table 25. Those strains which did not exactly fit into any of the recognised subspecies are also noted.

TABLE 25

Identities of 37 isolates of *B. melaninogenicus* from the
gingival crevice

| | Number of strains fitting the identification scheme (fig.(v)) | Number of strains not fitting the identification scheme (fig.(v)) |
|------------------------------------|--|--|
| <u><i>B. melaninogenicus</i></u> | | |
| ss. <u><i>melaninogenicus</i></u> | 10 | 10 |
| ss. <u><i>intermedius</i></u> | 13 | 2 |
| ss. <u><i>asaccharolyticus</i></u> | 2 | 0 |

The strains which did not fit the patterns of results in the identification scheme differed from those patterns thus:

| | |
|--|-----------|
| a) Penicillin resistant | 7 strains |
| b) Gelatin not liquefied | 4 strains |
| c) Penicillin resistant and gelatin not liquefied | 1 strain |

The 30 strains collected in Method 2 of the clinical sampling survey were identified as shown in Table 26 on the following page.

TABLE 26

Identities of 30 isolates of Gram-negative anaerobic bacilli from the gingival crevice

| Identification | Number of strains fitting the identification scheme (Fig.(v)) | Number of strains not fitting the identification scheme (Fig.(v)) |
|--|---|---|
| <u>B. melaninogenicus</u> | | |
| ss. <u>melaninogenicus</u> | 2 | 3 |
| ss. <u>intermedius</u> | 6 | 0 |
| ss. <u>asaccharolyticus</u> | 1 | 0 |
| <u>B. oralis</u> | 1 | 2 |
| <u>Bacteroides</u> sp. ¹ | 5 | 0 |
| <u>F. polymorphum</u> | 2 | 0 |
| <u>Fusobacterium</u> sp. ² | 0 | 3 |
| Strains that grew too ³ poorly to be identified | 0 | 3 |
| Unidentifiable | 0 | 1 |
| Aerobe ⁴ | 0 | 1 |

Notes relating to Table 26

- 1 = The 5 strains in this group were all identical in their reactions to the tests in the identification scheme. The results resembled those obtained with B. melaninogenicus ss. melaninogenicus except that pigment was not produced. Originally these strains were thought to be B. ochraceus but

they are strict anaerobes and differ morphologically from B. ochraceus. The pattern of results obtained with these strains is, however, similar to that obtained with B. ochraceus VPI 2845.

- 2 = These organisms have some characteristics of fusobacteria studied but do not exactly fit into any of the recognised patterns. One strain appeared to resemble L. buccalis morphologically and in some biochemical tests (i.e. fermentation) but did not exactly fit the pattern obtained with L. buccalis NCTC 10249 in the whole series of tests. Another strain was found to be motile and could be a Campylobacter sp. The results obtained with this strain (WPH 185) were compared with those given in the VPI Anaerobe Laboratory Manual (Holdeman and Moore, 1973 q.v.) but no similar pattern was recorded there.
- 3 = Three strains did not grow well on dye and bile-salt tolerance media and could not be identified.
- 4 = One strain was found to grow in O_2 with 10% CO_2 and to produce pitting of the agar surface and was provisionally identified as Eikenella corrodens.

The 5 strains that could be identified but which did not fit the identification scheme as given in fig. (v), differed from the expected patterns in the following manner:

B. melaninogenicus ss. melaninogenicus

- | | |
|--------------------------|-----------|
| a) Penicillin resistant | 1 strain |
| b) Gelatin not liquefied | 2 strains |

B. oralis

Failed to grow in

Victoria blue 4R

2 strains

SECTION B

THE ISOLATION OF ORAL GRAM-NEGATIVE ANAEROBIC BACILLI

Results of the preliminary survey of
clinical sampling methods

The recovery of B. melaninogenicus in this survey was poor. Out of the 100 subjects sampled only 41 yielded black-pigmented colonies on blood agar.

Samples removed with a curette (1-34) did not yield any strains of B. melaninogenicus. Those samples removed with a wooden stick (35-100) yielded most of the 41 strains on the directly inoculated plates. Subculture of these strains from the primary isolation plate resulted in the loss of seven strains and of the 34 strains that grew in pure subculture only 27 subsequently grew in cooked-meat broth.

Results of the experiments to develop improved media
for use in further clinical sampling surveys

a) Studies on transport media

The quantitative recovery of the two strains of B. melaninogenicus ss. intermedius (WPH 99 and NCTC 9338) from the transport media at time intervals up to 24 h was recorded graphically (see fig. (vi) and fig. (vii)). Both organisms showed an initial increase in numbers at 1 h in VMG II, Reduced Transport Fluid and Thioglycollate medium without dextrose or indicator. In Gordon's diluent, however, both organisms showed a decline from the initial count. The recovery of WPH 99 (the fresh clinical

isolate) declined rapidly in all transport media (fig. (vi)) and between 4 h and 6 h the count had fallen practically to zero in all media except VMG II. In VMG II the fall in bacterial count became less rapid at 4 h and small numbers were still recovered at 24 h.

The recovery of NCTC 9338 in Reduced Transport Fluid and Thioglycollate medium without dextrose or indicator declined at a similar rate from 1 h to 4 h (fig. (vii)). An unexplained rise occurred at 6 h in Reduced Transport Fluid, which was probably an aberrant result as no organisms were recovered later. There appeared to be a greater increase in numbers of this strain when held in VMG II than when held in other transport media. The recovery almost doubled at 6 h compared with the initial reading. At 24 h the recovery was similar to the initial reading. A better recovery of strains was obtained from VMG II than from the other transport media. Some aspects of the behaviour of these strains in transport media need further investigation (see discussion page 165) but for the planned clinical sampling survey VMG II was chosen as the transport medium.

b) Studies on liquid media

The growth of these test strains in the eight media selected is given in Table 27.

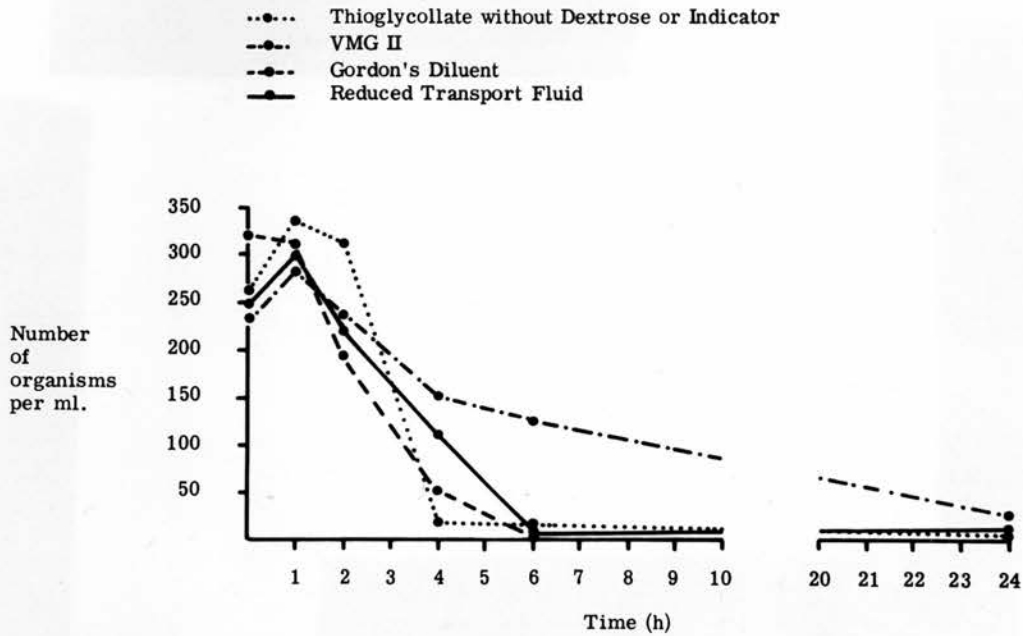
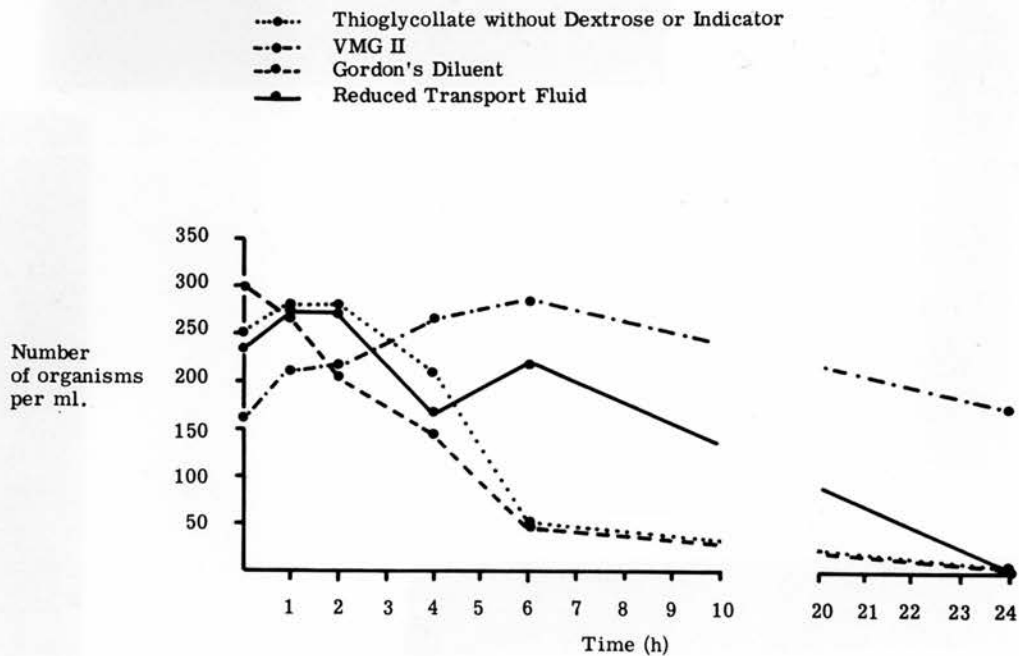
Figure (vi)The survival of *B. melaninogenicus* WPH 99 in four transport mediaFigure (vii)The survival of *B. melaninogenicus* NCTC 9338 in four transport media

TABLE 27

The growth of strains in liquid media

| Strain no. | CMB | Thio | BHI | Media used* | | | | 109 |
|---------------|-----|------|-----|-------------|----|-----|-----|-----|
| | | | | HDB | BM | MBM | MME | |
| 2296 | + | + | + | ↓ | + | + | + | - |
| 2/2009 | - | - | + | + | - | + | + | + |
| WPH 12 | + | + | + | + | + | + | + | + |
| WPH 32 | - | - | ↓ | - | ↓ | + | + | - |
| WPH 34 | - | + | ↓ | + | ↓ | + | + | - |
| WPH 36 | + | + | - | ↓ | + | + | + | + |
| WPH 38 | - | - | ↓ | ↓ | ↓ | + | + | - |
| WPH 42 | + | ↓ | + | ↓ | + | + | + | + |
| WPH 43 | + | + | - | ↓ | + | + | + | - |

* Key to media used (see also Appendix II):

- CMB - Robertson's cooked-meat broth
- Thio - thioglycollate medium with haemin and menadione
- BHI - brain-heart-infusion broth with haemin and menadione
- HDB - horse-digest broth with haemin and menadione
- BM - B.M. broth
- MBM - modified B.M. broth
- MME - modified medium E
- 109 - NCTC 109 TC medium
- - no growth detected microscopically

Key to Table 27 (continued)

- 1 - some growth detected on wet-film; no obvious turbidity
- +
- adequate growth; obvious turbidity

Although most media were seen to be an improvement on Robertson's cooked-meat broth for the growth of these demanding strains, in only two media, modified B.M. broth and modified medium E, did all organisms grow well. Because of its easier method of preparation modified B.M. broth was selected for use in the clinical sampling survey.

c) Studies on solid media

The recovery of B. melaninogenicus ss. intermedius NCTC 9338 on the two solid media in experiment (1) was determined.

The average number of colonies on plates of lysed-blood agar was 410.1 and on plates of modified medium 10 - sucrose-blood agar was 410.9. Thus both media gave the same viable count of 4.1×10^8 cfu per ml. The total cell count determined microscopically was 7.8×10^8 cfu per ml. The colonies on lysed-blood agar were 1-2 mm diameter and black whereas those on modified medium 10 - sucrose-blood agar were pin-point and translucent.

The average colony count of the fresh clinical isolate of B. melaninogenicus ss. intermedius WPH 99 on three media; lysed-blood agar with menadione, modified medium 10-sucrose-blood agar and modified B.M.-blood agar is given in Table 28.

TABLE 28

Average colony count of *B. melaninogenicus* ss. *intermedius* (WPH 99)
on three solid media

| Medium* | Average colony count in jar | | | | | | Average colony count | Viable count cfu/ml |
|---------|-----------------------------|-----|-----|-----|-----|-----|----------------------|------------------------|
| | 1 | 2 | 3 | 4 | 5 | 6 | | |
| 1) LBA | 137 | 147 | 143 | 141 | 154 | 177 | 150 | 7.5×10^8 |
| 2) MM10 | 181 | 200 | 158 | 177 | 179 | 151 | 174 | 8.7×10^8 |
| 3) BM | 192 | 192 | 185 | 152 | 198 | 193 | 185 | 9.25×10^8 |

* Key to media used:

- LBA - lysed-blood agar with menadione
 MM10 - modified medium 10-sucrose-blood agar
 BM - modified B.M.-blood agar

The total microscopic count was 1.06×10^9 cfu per ml. Again the colonies of WPH 99 on Modified medium 10-sucrose-blood agar were not black-pigmented but colonies of the other two media did produce black pigment and were somewhat larger in diameter than the colonies on modified medium 10-sucrose-blood agar.

Because the black-pigmented colonies would be more easily distinguished in mixed culture, modified B.M.-blood agar was thought to be of more practical use in future clinical sampling surveys than modified medium 10-sucrose-blood agar.

d) Studies on selective media

Growth in the two selective media was recorded for the list of organisms given in Tables 9 and 10 and for the five organisms of other genera commonly encountered in the mouth (see Materials and Methods page 62). The growth of the strains was recorded as -, 1, +, ++ or +++ depending on the extent of growth seen on the plate, see fig. (iii). These results are shown in Table 29.

TABLE 29The growth of strains on selective media

| Organism** | Selective medium* | | Basal medium* |
|------------|-------------------|------------------------|---------------|
| | kanamycin | kanamycin & vancomycin | control |
| VPI 4196 | +++ | +++ | +++ |
| ATCC 15930 | +++ | +++ | +++ |
| 30 | +++ | +++ | +++ |
| 9336 | +++ | +++ | +++ |
| 9337 | +++ | +++ | +++ |
| 7CM | +++ | +++ | +++ |
| B20-s | ++ | + | ++ |
| NCTC 10562 | + | + | ++ |
| WPH 68 | +++ | +++ | +++ |
| WPH 88 | +++ | +++ | +++ |
| WPH 99 | +++ | +++ | +++ |
| 2296 | +++ | +++ | +++ |

TABLE 29 (continued)

| Organism** | Selective medium* | | Basal medium* control |
|--------------------------------|-------------------|---------------------------|--------------------------|
| | kanamycin | kanamycin & vancomycin | |
| 3502 | + | + | ++ |
| WPH 89 | ++ | ++ | ++ |
| WPH 41 | ++ | ++ | +++ |
| WPH 45 | ++ | ++ | +++ |
| WPH 94 | + | + | +++ |
| WPH 113 | ++ | ++ | +++ |
| WPH 114 | +++ | +++ | +++ |
| <u>"Strept. viridans"</u> | - | - | ++ |
| <u>Staph. albus</u> | - | - | + |
| <u>Corynebacterium xerosis</u> | - | - | + |
| <u>Neisseria catarrhalis</u> | - | - | - |
| <u>E. coli</u> | - | - | +++ |

Key to Table 29:

- * = for details of media see Materials and Methods page 63 ,
and Appendix II.
- ** = for details of strains see Materials and Methods, Tables
9 and 10.

e) Studies on enrichment media

Growth in the enrichment medium was recorded for the
strains listed in Tables 9 and 10 and for the five organisms of

other genera commonly encountered in the human mouth (see Materials and Methods page 62). After incubation the broths were used to inoculate modified B.M.-blood agar plates and the degree of growth was recorded as: -, \perp , +, ++, or +++ (see fig. (iii)). These results are shown in Table 30.

TABLE 30

The growth of strains in the enrichment broth

| Organism | Degree of growth | Organism | Degree of growth |
|------------|------------------|--------------------------------|------------------|
| VPI 4196 | +++ | 3502 | \perp |
| ATCC 15930 | ++ | WPH 89 | \perp |
| 30 | +++ | WPH 41 | + |
| 9336 | +++ | WPH 45 | +++ |
| 9337 | + | WPH 94 | \perp |
| 7CM | +++ | WPH 113 | ++ |
| B20-a | ++ | WPH 114 | ++ |
| NCTC 10562 | +++ | <u>"Strept. viridans"</u> | \perp |
| WPH 68 | +++ | <u>Staph. albus</u> | - |
| WPH 88 | +++ | <u>Corynebacterium xerosis</u> | - |
| WPH 99 | +++ | <u>Neisseria catarrhalis</u> | - |
| 2296 | +++ | <u>E. coli</u> | - |

Results of the clinical sampling survey

The degree of disease assessed for each of the 50 patients in the survey is indicated in Table 31.

TABLE 31

The degree of disease of patients in the survey

| Degree of disease (Russell's Index) | Number of patients |
|--|--------------------|
| 0 | 5 |
| 1 | 16 |
| 2 | 18 |
| 4 | 4 |
| 6 | 7 |
| 8 | 0 |

The results of the attempted isolation of B. melaninogenicus by each route and for the 50 patients is shown in Table 32. Each route of isolation could produce one of three results; a) growth in pure subculture (G); b) black colonies seen but lost on subculture (L); c) no black colonies seen (N). These results are indicated by an asterisk (*) in Table 32 for each method of isolation and for each patient.

Key to Table 32

G = B. melaninogenicus strain isolated in
pure subculture

| | |
|-------------------|---|
| L | = black colony seen but lost on subculture |
| N | = no black colony seen on primary plate |
| NS | = non-selective medium |
| K | = selective medium containing kanamycin |
| KV | = selective medium containing kanamycin and vancomycin |
| DIRECT | = samples directly plated |
| TRANSPORT | = samples held in transport medium |
| ENRICHMENT (24 h) | = samples held in enrichment medium for 24 h |
| E2 (48 h) | = samples held in enrichment medium for 48 h |

These data together with the degree of disease recorded for each patient were analysed by Mrs. Margaret Shotter of the Department of Statistics of the University of Edinburgh. Analyses of these data were performed on the recovery of strains from each individual patient so as to remove the inter-patient variation. The total number of cases in which the organisms grew in pure subculture are shown in a histogram (figure viii) from which it is easier to see the differences between the results obtained with the isolation techniques. Each column represents a route of isolation and the hatched area of each column indicates the number of samples that grew in pure subculture. Above this section of each column is an unhatched portion which indicates the number of organisms seen but lost on subculture. The total height of the column indicates the incidence of black or brown-pigmented colonies. Those cases in which no black or brown colonies were seen can be calculated by subtracting the incidence figure from 50 (the number of patients

Figure (viii)

Histogram illustrating the number of samples yielding *B. melaninogenicus* strains in relation to the primary isolation procedure used in the survey of 50 patients

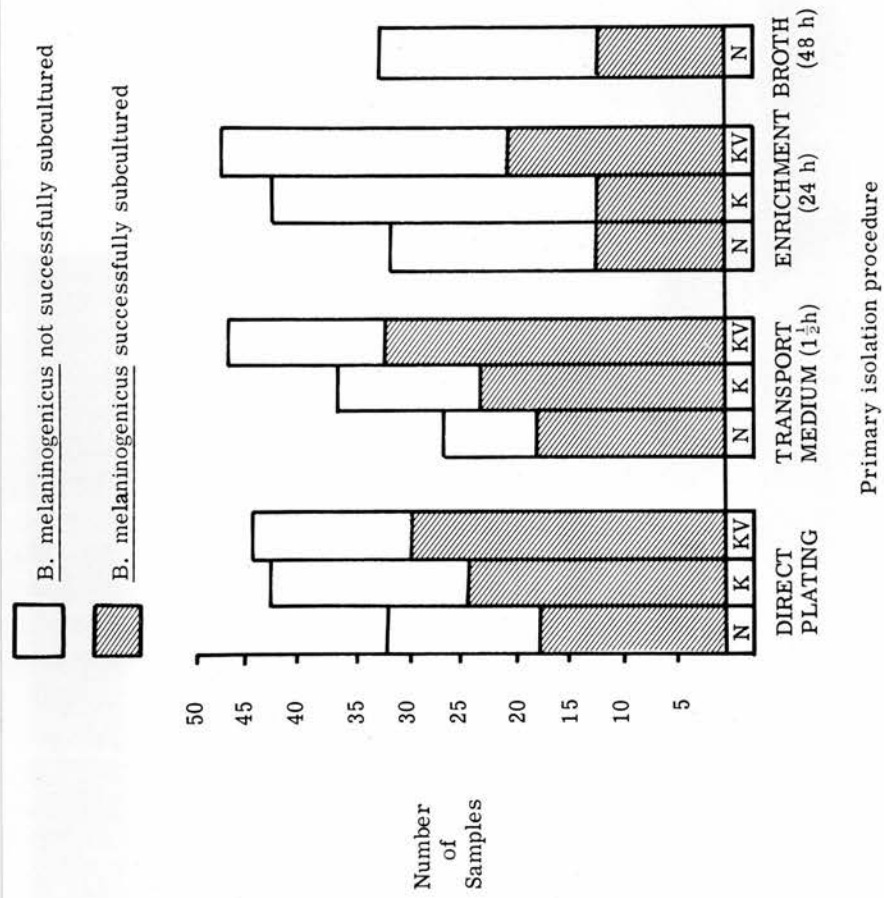


TABLE 32

Results of the attempted isolation of *B. melaninogenicus*
strains by each route and for each patient in the survey
of 50 patients (for key see text page 135)

| PATIENT | DIRECT | | | | | | | | | TRANSPORT | | | | | | | | | ENRICHMENT (24 h) | | | | | | | | | E2 (48 h) | | |
|---------|--------|----|----|----|----|---|----|----|---|-----------|---|----|----|----|----|----|----|---|-------------------|----|----|----|----|---|----|----|---|-----------|----|----|
| | NS | | | K | | | KV | | | NS | | | K | | | KV | | | NS | | | K | | | KV | | | NS | | |
| | G | L | N | G | L | N | G | L | N | G | L | N | G | L | N | G | L | N | G | L | N | G | L | N | G | L | N | G | L | N |
| 201 | | * | | | * | | | * | | | * | | | * | | | * | | | * | | | * | | | * | | | * | |
| 202 | | * | | | * | | | * | | | * | | | * | | | * | | | * | | | * | | | * | | | * | |
| 203 | | * | | | * | | | * | | | * | | | * | | | * | | | * | | | * | | | * | | | * | |
| 204 | | * | | | * | | | * | | | * | | | * | | | * | | | * | | | * | | | * | | | * | |
| 205 | * | | | * | | | | * | | | * | | | * | | | * | | | * | | | * | | | * | | | * | |
| 206 | * | | | * | | | | * | | | * | | | * | | | * | | | * | | | * | | | * | | | * | |
| 207 | * | | | * | | | | * | | | * | | | * | | | * | | | * | | | * | | | * | | | * | |
| 208 | * | | | * | | | | * | | | * | | | * | | | * | | | * | | | * | | | * | | | * | |
| 209 | * | | | * | | | | * | | | * | | | * | | | * | | | * | | | * | | | * | | | * | |
| 210 | * | | | * | | | | * | | | * | | | * | | | * | | | * | | | * | | | * | | | * | |
| 211 | | * | | | * | | | * | | | * | | | * | | | * | | | * | | | * | | | * | | | * | |
| 212 | | * | | | * | | | * | | | * | | | * | | | * | | | * | | | * | | | * | | | * | |
| 213 | | * | | | * | | | * | | | * | | | * | | | * | | | * | | | * | | | * | | | * | |
| 214 | | * | | | * | | | * | | | * | | | * | | | * | | | * | | | * | | | * | | | * | |
| 215 | | * | | | * | | | * | | | * | | | * | | | * | | | * | | | * | | | * | | | * | |
| 216 | * | | | * | | | | * | | | * | | | * | | | * | | | * | | | * | | | * | | | * | |
| 217 | * | | | * | | | | * | | | * | | | * | | | * | | | * | | | * | | | * | | | * | |
| 218 | * | | | * | | | | * | | | * | | | * | | | * | | | * | | | * | | | * | | | * | |
| 219 | * | | | * | | | | * | | | * | | | * | | | * | | | * | | | * | | | * | | | * | |
| 220 | * | | | * | | | | * | | | * | | | * | | | * | | | * | | | * | | | * | | | * | |
| 221 | * | | | * | | | | * | | | * | | | * | | | * | | | * | | | * | | | * | | | * | |
| 222 | * | | | * | | | | * | | | * | | | * | | | * | | | * | | | * | | | * | | | * | |
| 223 | * | | | * | | | | * | | | * | | | * | | | * | | | * | | | * | | | * | | | * | |
| 224 | * | | | * | | | | * | | | * | | | * | | | * | | | * | | | * | | | * | | | * | |
| 225 | * | | | * | | | | * | | | * | | | * | | | * | | | * | | | * | | | * | | | * | |
| 226 | * | | | * | | | | * | | | * | | | * | | | * | | | * | | | * | | | * | | | * | |
| 227 | * | | | * | | | | * | | | * | | | * | | | * | | | * | | | * | | | * | | | * | |
| 228 | * | | | * | | | | * | | | * | | | * | | | * | | | * | | | * | | | * | | | * | |
| 229 | * | | | * | | | | * | | | * | | | * | | | * | | | * | | | * | | | * | | | * | |
| 230 | * | | | * | | | | * | | | * | | | * | | | * | | | * | | | * | | | * | | | * | |
| 231 | * | | | * | | | | * | | | * | | | * | | | * | | | * | | | * | | | * | | | * | |
| 232 | * | | | * | | | | * | | | * | | | * | | | * | | | * | | | * | | | * | | | * | |
| 233 | * | | | * | | | | * | | | * | | | * | | | * | | | * | | | * | | | * | | | * | |
| 234 | * | | | * | | | | * | | | * | | | * | | | * | | | * | | | * | | | * | | | * | |
| 235 | * | | | * | | | | * | | | * | | | * | | | * | | | * | | | * | | | * | | | * | |
| 236 | * | | | * | | | | * | | | * | | | * | | | * | | | * | | | * | | | * | | | * | |
| 237 | * | | | * | | | | * | | | * | | | * | | | * | | | * | | | * | | | * | | | * | |
| 238 | * | | | * | | | | * | | | * | | | * | | | * | | | * | | | * | | | * | | | * | |
| 239 | * | | | * | | | | * | | | * | | | * | | | * | | | * | | | * | | | * | | | * | |
| 240 | * | | | * | | | | * | | | * | | | * | | | * | | | * | | | * | | | * | | | * | |
| 241 | * | | | * | | | | * | | | * | | | * | | | * | | | * | | | * | | | * | | | * | |
| 242 | * | | | * | | | | * | | | * | | | * | | | * | | | * | | | * | | | * | | | * | |
| 243 | * | | | * | | | | * | | | * | | | * | | | * | | | * | | | * | | | * | | | * | |
| 244 | * | | | * | | | | * | | | * | | | * | | | * | | | * | | | * | | | * | | | * | |
| 245 | * | | | * | | | | * | | | * | | | * | | | * | | | * | | | * | | | * | | | * | |
| 246 | * | | | * | | | | * | | | * | | | * | | | * | | | * | | | * | | | * | | | * | |
| 247 | * | | | * | | | | * | | | * | | | * | | | * | | | * | | | * | | | * | | | * | |
| 248 | * | | | * | | | | * | | | * | | | * | | | * | | | * | | | * | | | * | | | * | |
| 249 | * | | | * | | | | * | | | * | | | * | | | * | | | * | | | * | | | * | | | * | |
| 250 | * | | | * | | | | * | | | * | | | * | | | * | | | * | | | * | | | * | | | * | |
| TOTAL | 17 | 15 | 18 | 24 | 19 | 7 | 29 | 16 | 5 | 18 | 9 | 23 | 23 | 14 | 13 | 32 | 15 | 3 | 12 | 19 | 19 | 12 | 30 | 8 | 20 | 27 | 3 | 12 | 21 | 17 |

in the survey). This is indicated by the height above the columns in figure (viii).

No correlation was noted between the degree of disease recorded and the success of any particular method of isolating B. melaninogenicus strains in either pure or mixed culture. The correlation coefficient was -0.13.

The statistical analysis revealed no significant differences between results obtained by direct plating, transport and enrichment-broth procedures for samples cultured on the same solid medium. Highly significant differences were noted in the recovery^{of}/strains from the different solid media. The poorest rate of isolation in pure culture was found on samples grown on non-selective media and there was a highly significant increase in the recovery of B. melaninogenicus strains when the selective medium contained kanamycin and vancomycin. This medium also gave a significantly higher recovery than the medium containing kanamycin alone.

An impression of the improved isolation of B. melaninogenicus strains from the medium containing kanamycin and vancomycin can be seen in plates XV and XVI. Plate XV shows the appearance after 48 h incubation of the first lines of streaking out from the well of a plate of modified B.M.-blood agar inoculated with 0.02 ml of a gingival sample suspended in VMG II transport medium. Plate XVI shows the appearance after 48 h incubation of the first lines of streaking out from the well of a plate of modified B.M.-blood agar containing kanamycin and

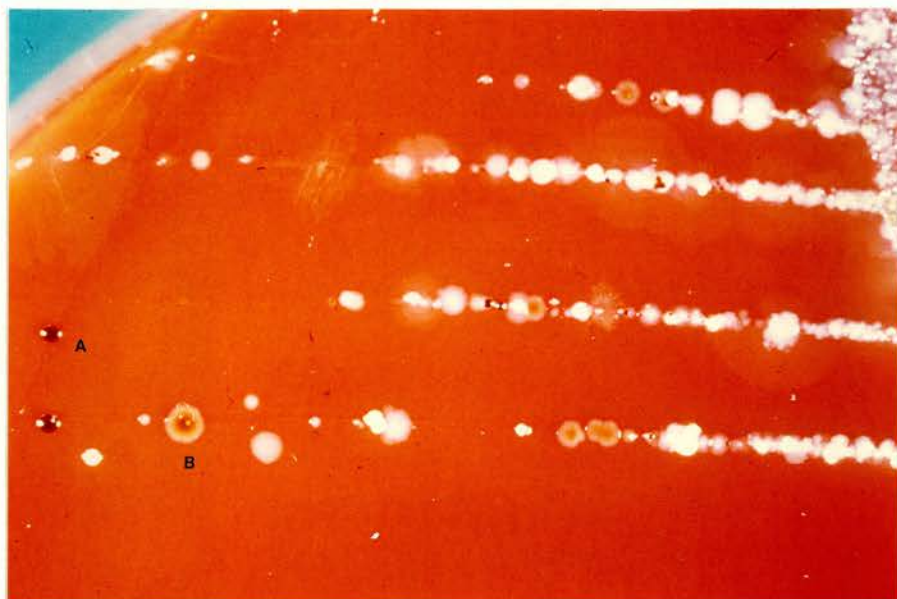
vancomycin which was inoculated with 0.02 ml of the same sample.

A typical colony of B. melaninogenicus ss. intermedius is indicated by "A" and a typical colony of B. melaninogenicus ss. melaninogenicus is indicated by "B".

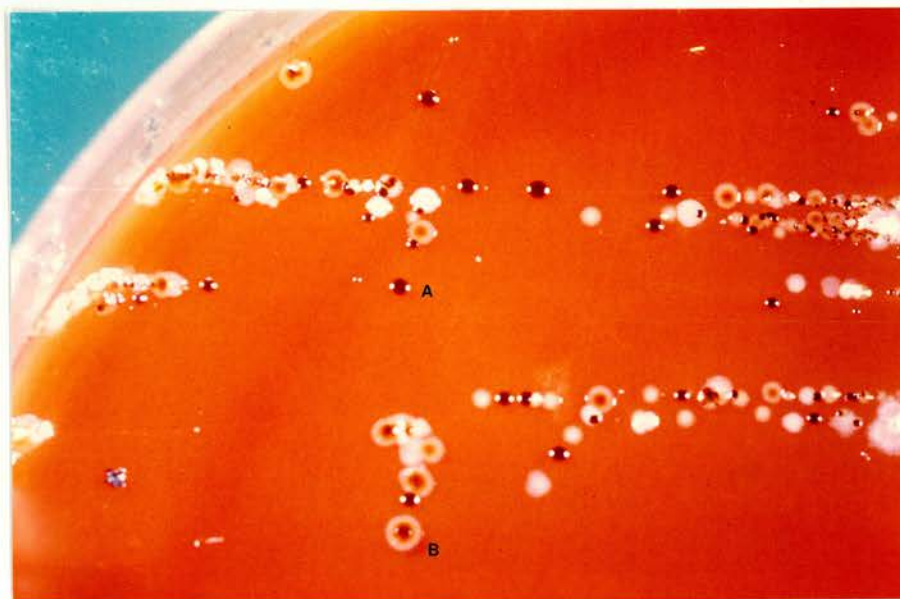
From the study of samples from five patients a wide range of Gram-negative, non-sporing, anaerobic bacilli was isolated. The identities of these strains are given in Table 26. B. fragilis strains were not isolated from these samples and the most frequently isolated organisms were B. melaninogenicus ss. intermedius and ss. melaninogenicus. F. polymorphum and B. oralis were also recognised and a number of organisms were isolated that could only be identified at the genus level as Bacteroides spp. and Fusobacterium spp. The five strains listed in Table 26 as Bacteroides sp. all gave identical results in the tests for identification and resembled B. melaninogenicus ss. melaninogenicus except that black pigment was not produced.

Plate XV

The appearance of a gingival sample cultured on modified B.M.-blood agar (see text for details, page 139)

Plate XVI

The appearance of an aliquot of the above gingival sample cultured on modified B.M.-blood agar containing kanamycin and vancomycin (see text for details, page 139)



DISCUSSION

Members of the family Bacteroidaceae have been increasingly recognised as potential pathogens in man and animals and have been associated with disease in a variety of sites. The infectivity of some of these organisms has been demonstrated in experimental animals (see Introduction chapter, page 22). Advances in the isolation, culture and identification of these organisms have led to an increasing awareness of their importance.

There remains, however, a need for relatively prompt identification in the clinical laboratory. Infection with Gram-negative, non-sporing, anaerobic bacilli is usually mixed and in consequence it is important for the clinical laboratory to be able to detect when such organisms are present in a specimen and whether or not they are pathogens or merely representative of the anaerobic commensal flora. B. fragilis ss. fragilis is known to be associated more frequently with disease states than either B. fragilis ss. vulgatus or B. fragilis ss. thetaiotaomicron (Smith, 1975). Knowledge of the relative frequency of involvement of members of the Bacteroidaceae in pathogenic roles depends upon further developments in isolation, culture and detailed characterisation. Accordingly, in this chapter consideration will be given to: i) methods of anaerobic incubation; ii) a review of methods of identifying Gram-negative, non-sporing, anaerobic bacilli; iii) methods adopted for identifying and classifying the organisms in the present study; and iv) methods of isolating Gram-negative, non-sporing anaerobes with special reference to their isolation from the mouth.

Methods of anaerobic incubation

It has been claimed that the use of an anaerobic cabinet (Drasar, 1967; Aranki et al., 1969) gives an improved recovery of anaerobic organisms. Problems exist, however, in the handling of specimens in a cabinet or glove-box and these are overcome by the use of roll-tubes in the system devised by Hungate (1950). This method has been adopted by Cato et al. (1970). McMinn and Crawford (1970), Gordon, Stutman and Loesche (1971) and Dowell (1972) found that roll-tubes gave better recovery of anaerobes than their versions of 'conventional' anaerobic jar procedures. Roll-tubes used outside a cabinet with an anaerobic gassing device, are easier to handle than plates inside a cabinet; but inoculation to achieve discrete colonies and picking of these colonies for subculture is difficult. These handling problems are especially apparent in the busy clinical laboratory where these methods are expensive in terms of labour. Pre-reduced anaerobically sterilised media were reported to give improved recovery of anaerobes (McMinn and Crawford, 1970; Dowell, 1972). Starr (1974), however, did not find any improvement in recovery when he compared pre-reduced anaerobically sterilised media with freshly prepared media. Freshly prepared media have also been advocated by Watt (1972a).

In a study of the oxygen sensitivity of various anaerobic organisms Loesche (1969) noted that the Bacteroidaceae were moderate anaerobes capable of surviving exposure to oxygen in the room for 90 min. without loss of viability. Tally et al. (1975) have shown that fresh clinical anaerobic organisms, including

B. fragilis, B. melaninogenicus, B. oralis and fusobacteria, can tolerate oxygen for up to 8 h in the air. This supports the views held in this laboratory that anaerobic jar procedures, if properly conducted, are sufficient for the growth of Gram-negative anaerobic bacilli (Collee, Rutter and Watt, 1971; Watt, 1972b; Watt, 1973; Watt, Hoare and Collee, 1973). In a comparison of anaerobic cabinet and standardised anaerobic jar procedures on the quantitative recovery of anaerobes from faeces, Watt, Collee and Brown (1974) found no difference in the recovery of these organisms by the two methods. Confirmation of these findings has been made by Rosenblatt, Fallon and Finegold (1973) who compared Gas-Pak jars, evacuation and replacement jar procedures and the roll-tube method. These workers found no improvement in the recovery of anaerobes from the clinical specimens they examined when roll tubes with pre-reduced anaerobically sterilised media were used. Starr (1974) noted no difference in the recovery of anaerobes from fifteen abscesses cultured in roll-tubes, in an anaerobic cabinet or by conventional methods using the Gas-Pak.

The Gas-Pak modification of the conventional anaerobic jar procedure is of use in clinical laboratories and has been shown to allow growth of very exacting anaerobes such as Clostridium oedematiens type D (Collee et al., 1972). A series of 700 paired clinical specimens were compared in an anaerobic cabinet procedure and a conventional jar technique using the Gas-Pak (Spaulding et al., 1974). Although the continuous anaerobic handling and anaerobic cabinet gave a better recovery

of anaerobes, these authors attributed the poorer performance of the jars and Gas-Pak to ineffective catalysts and prolonged exposure of the specimen to air. Having rectified these faults, the results with the Gas-Pak were comparable with those obtained with the anaerobic cabinet. Ferguson, Philips and Tearle (1975) noted a problem with the carbon dioxide generator in Gas-Pak sachets in that the borohydride used to produce hydrogen caused a rise in pH which lowered the amount of CO_2 released by the action of the water on the citrate-bicarbonate mixture. This undoubtedly presents a problem as CO_2 has been shown to be an important growth factor for Gram-negative, non-sporing anaerobes (Watt, 1973).

A review of methods of identifying Gram-negative non-sporing anaerobic bacilli

Much confusion has existed in the classification of Gram-negative, non-sporing, anaerobic bacilli. A number of clearly different organisms have been given the same name and similar organisms have been given different names. Some examples of the early classification of these organisms are given in the Introduction to this thesis; a summary of some family and generic names that have been used for these organisms is given in Table 33. The names used in this study are largely those adopted by Holdeman and Moore in Bergey's Manual of Determinative Bacteriology, 1974.

A wide variety of approaches have been adopted in order to identify these organisms and it has not always been clear at

which taxonomic level these various approaches have operated. Many methods of identification involve difficult or time-consuming procedures which make them unsuitable for use in a clinical laboratory. The use of so many tests for the identification of these organisms is, in part, responsible for the confusing taxonomy.

Much early work depended largely on morphological characteristics of the organisms, viewed microscopically, or on colonial morphology when culture was possible (Macdonald, 1953; Sebald, 1962). Many biochemical tests have been used to identify these organisms. Jackins and Barker (1951) investigated fermentative processes in fusobacteria, and other biochemical tests were performed on fusiforms by Macdonald (1953); Omata and Braunberg (1960); Hadi and Russell (1968a) and Werner Neuhaus and Hussels (1971). Biochemical properties of B. melaninogenicus were investigated by Sawyer, Macdonald and Gibbons (1962) and the reaction of bacteroides organisms in general were studied by Prévot (1966 a and b) and James and Robinson (1975). Funderburk and Kester (1975) noted the value of valine, malic and pyruvic dehydrogenases in the differentiation of bacteroides. B. melaninogenicus has received considerable attention and identification has been based on pigment production, and a number of biochemical tests (Williams et al., 1974 and 1975; Duerden, 1975; Harding, Sutter and Finegold, 1975).

The analysis of the acid end-products of glucose fermentation by gas-liquid chromatography has been employed in the

TABLE 33

Family and generic names that have been used for
Gram-negative, non-sporing, anaerobic bacteria
(partly after Aalbaek, 1973)

[illegible]

identification of Gram-negative, non-sporing anaerobes. Werner (1969) first described the method for demonstrating that B. convexus was a similar organism to B. fragilis. Cato et al. (1970) and Moore (1970) noted the use of acid end-product analysis in the identification of Bacteroidaceae. The results of acid end-product analysis have been used in conjunction with the results of more conventional biochemical tests to identify Gram-negative, non-sporing anaerobes (Loesche and Gibbons, 1965; Barnes and Goldberg, 1968; Werner, Pulverer and Reichertz, 1971; Werner, 1974; Harding, Sutter and Finegold, 1975; Williams et al., 1974 and 1975). A number of useful laboratory manuals have been produced recently in which the results of biochemical tests and gas-liquid chromatographic analysis are used to identify a wide range of anaerobes (Cato et al., 1970; Sutter et al., 1972; Holdeman and Moore, 1973; Sutter, Vargo and Finegold, 1975).

Identification of these organisms at a less differentiated level has been based on tolerance tests using: (i) antibiotic resistance (Finegold, Harada and Miller, 1967; Sutter and Finegold, 1971; Werner, 1972 and Peach, 1975); (ii) the effect of dyes on growth (Baird-Parker, 1957; Suzuki, Ushijima and Ichinose, 1966); and (iii) sensitivity to bile salts (Shimada, Sutter and Finegold, 1970).

Ultraviolet red-fluorescence has been noted in B. melaninogenicus (Myers et al., 1969) and may be useful for the early identification of this organism as the observation of fluorescence can be made before black-pigment is produced in

the colony.

The ratio of the DNA bases guanine and cytosine indicate fundamental similarities or differences between bacteria. This property has been studied by Sebald (1962); Sebald and Véron (1963) and Williams et al. (1974 and 1975). The analysis of DNA homologies has been studied by Johnson (1973). Cell wall analyses of B. melaninogenicus strains have been made by Williams et al. (1974 and 1975).

A large number of characteristics have been studied for numerical taxonomic studies of the bacteria in the family Bacteroidaceae by Barnes and Goldberg (1968) and Sundqvist (1976).

Other methods found useful to identify members of the Bacteroidaceae have included:

- (i) the separation of proteins by gel electrophoresis to differentiate Leptotrichia spp. from Fusobacterium spp. (Baboolal, 1972) and in the identification of Bacteroides species (Strom et al., 1976);
- (ii) Serological grouping of Bacteroides spp. particularly from the rumen (Sharpe, 1971) and B. melaninogenicus (Lambe, 1974); and
- (iii) The production of bacteriocin-like substances by some sub-species of B. fragilis (Podhaisky and Reinhold, 1970; Collee, Watt and Dewhurst, 1974; Watt and Collee, 1974).

Methods adopted for identifying and classifying
the organisms in the present study

Details of the methods adopted are given in the Materials and Methods chapter (Section A). The tests fell into three groups; tolerance tests, biochemical tests and antibiotic-disk-resistance tests. Attempts were made to adapt each test to the conditions of culture imposed by the growth requirements of these organisms. Control organisms belonging to the same family were used for all tests and these were thought to be more meaningful than the aerobic controls used routinely in clinical microbiological laboratories. Problems of growth with some exacting strains were experienced and later work on the development of improved media represent attempts to overcome these problems.

In the first series of experiments the 165 strains listed in Appendix Ia fell into three broad groups: (i) non-pigmented B. fragilis-like organisms; (ii) pigmented B. melaninogenicus-like strains; (iii) Fusobacterium-like strains.

Biochemical tests

The biochemical tests differentiated between the five recognised subspecies of B. fragilis and the patterns obtained with the remaining B-strains showed clusters of strains related to these reference strains with a number of intermediate patterns. B. oralis and B. ochraceus gave similar patterns to other B-strains in the biochemical tests.

Two groups of B. melaninogenicus strains were noted that

were differentiated on the ability of one group to ferment carbohydrates. The saccharolytic strains were related to reference strains of B. melaninogenicus ss. intermedius. Only one strain of B. melaninogenicus ss. melaninogenicus was included in these organisms (VPI 4196) although B. melaninogenicus ATCC 15930 (formerly B. oralis) was found to have a similar, but not identical, pattern of results.

It was thought necessary to isolate further strains of B. melaninogenicus ss. melaninogenicus before the taxonomic position of this subspecies could be assessed. B. melaninogenicus ss. asaccharolyticus was clearly different from the saccharolytic strains of B. melaninogenicus on the basis of biochemical tests. Asaccharolytic strains of B. melaninogenicus have been considered by the International Committee of Systematic Bacteriology, Taxonomic Subcommittee on Gram-negative anaerobic rods and may well be assigned to a separate species (Dr. Ella M. Barnes, personal communication).

Although all F-strains were clearly different from B-strains and M-strains they formed a heterogeneous group with considerable differences in biochemical characteristics within the group.

Tolerance tests

A broad separation of all 165 strains into three main groups was achieved with the tolerance tests. The B. fragilis strains gave similar patterns but B. fragilis ss. vulgatus strains

(except B. fragilis ss. vulgatus ATCC 8482) could be distinguished by their unique pattern. Most M-strains gave a single tolerance pattern but although this distinguished the M-strains from most of the B-strains a few exceptions existed, such as B. oralis NP 333, B. ochraceus VPI 2845 and a number of strains resembling B. fragilis according to the results of biochemical tests.

The reference strains in the F-strain group gave distinct tolerance patterns from both the B-strain and M-strain groups. A number of clinical isolates, however, gave tolerance patterns similar to those obtained with M-strains.

Antibiotic-disk-resistance tests

Good separation of the test strains was obtained with the antibiotic-disk-resistance tests. B. oralis NP 333, and B. ochraceus VPI 2845, which gave biochemical patterns similar to B. fragilis strains, produced antibiotic-disk-resistance patterns more closely resembling those of B. melaninogenicus strains. Sutter and Finegold (1971) suggested that disks containing specified concentrations of colistin, erythromycin, kanamycin, neomycin, penicillin and rifampicin could be used for the preliminary identification of Gram-negative, non-sporing anaerobic bacilli, but significant numbers of strains of B. fragilis and B. melaninogenicus gave atypical results with a number of these antibiotics.

It should be noted that the antibiotic-disk-resistance tests were used as a means of identifying strains and the results do not indicate the relative therapeutic value of these antibiotics.

The tests were performed under conditions that gave a good growth of test strains in an atmosphere which contained 10% CO₂. This is known to reduce the inhibition zone diameters around disks containing pH-sensitive antibiotics such as erythromycin and lincomycin (Ingham et al., 1970). Other factors related to the medium may also affect the zone diameters of these antibiotics (Watt and Brown, 1975) but although these factors will affect the clinical application of sensitivity tests they do not affect the results of resistance tests used for identification purposes. For this reason the term "resistance-test" is preferred to "sensitivity-test" as the latter implies a clinical application of the results which the tests do not justify.

The disk-resistance tests used an anaerobically incubated Oxford Staphylococcus as control and this may be of doubtful value. B. fragilis NCTC 9343 was also used as a control in an attempt to provide an anaerobic organism analogous to the Oxford Staphylococcus. Although strain NCTC 9343 has the merit of rapid growth it is resistant to many antibiotics and its value as a control in clinical sensitivity testing is less certain. The widely differing rates of growth of these test strains prevented the adoption of a standard incubation period. Zone diameters surrounding disks were therefore measured as soon as growth was readily visible.

Correlations

Tables 18-23 inclusive illustrate the correlation between the results obtained with biochemical tests and tolerance tests and that between biochemical tests and antibiotic-disk-resistance tests.

Dye-tolerance tests (Suzuki, Ushijima and Ichinose, 1966), bile-tolerance tests (Shimida, Sutter and Finegold, 1970) and antibiotic-disk-resistance tests (Finegold, Harada and Miller, 1967) have been used for preliminary identification of the Bacteroidaceae. A number of strains in this study would have been incorrectly identified had only one of these methods been adopted. The correlation between results obtained in the three approaches with these 165 strains was taken to indicate that a combined set of tests would produce a means of preliminary identification which would remove a number of anomalous results obtained by any single approach. Accordingly a short series of tests as shown in figure (v) was selected for their discriminating value.

The tests listed in figure (v) will identify the major sub-species of B. fragilis and B. melaninogenicus. Details of the procedures for carrying out these tests and the controls used are important particularly with reference to the reproducibility of results. After the results of the tests have been collected care must be taken not to use the tests as a sequential key. Only when the full list of results to the tests listed is used should an organism be assigned to the appropriate group and the occurrence of atypical results will then become clear. Strains of B. melaninogenicus are often slow to produce pigment although the

use of lysed blood in the medium accelerates its production. The results obtained with antibiotic-disk-resistance and tolerance tests will serve for confirmation of the identity of B. melaninogenicus strains which have produced pigment and these tests are important in identifying strains which are slow to produce pigment.

Whilst these tests are not based on any fundamental properties of the organisms, such as cell-wall composition or DNA base ratios, the tests used do give satisfactory separation of reference strains of the recognised species and subspecies of B. fragilis and B. melaninogenicus. Further strains of B. melaninogenicus ss. melaninogenicus, B. oralis, B. ochraceus, B. fragilis ss. distasonis and ss. ovatus are needed in order to confirm the value of the identification scheme given in figure (v). Similarly a number of reference strains of fusobacteria are easily recognisable from the results obtained in these tests but a number of clinical isolates were tested which did not resemble the pattern obtained with any of the recognised species of Fusobacterium. Smith (1975) noted that B. fragilis ss. distasonis and ss. ovatus are uncommon findings in clinical specimens and Holdeman and Moore (1974) stress the need for further work on these subspecies. They conclude that the B. fragilis-like organisms are a continuum of variants with clusters of strains which have been designated subspecies.

Consideration of the results obtained with strains of
Gram-negative non-sporing anaerobes
listed in Appendix Ib and Ic

The organisms listed in Appendix Ib were used to test the usefulness of the identification scheme (figure (v)) and those strains listed in Appendix Ic were then identified on the basis of results with tests in the identification scheme. From the identification of the oral isolates listed in Appendix Ic it was hoped to gain some knowledge of the occurrence of the various species and subspecies of Gram-negative, non-sporing anaerobes in the mouth.

There were 53 strains in the series listed in Appendix Ib which were identified according to the full series of tolerance, biochemical and antibiotic-disk-resistance tests. The results obtained in the tests listed in figure (v) were extracted and used to identify the organisms in accordance with the patterns of results in figure (v). One strain was found to be an aerobe, but of the 52 remaining strains 42 could be identified by the scheme (figure (v)). In the full series of tests anomalous results were more apparent and only 29 strains exactly fitted patterns similar to those obtained with the first 165 strains.

The 10 strains which could not be identified by the scheme included one reference strain, Sphaerophorus varians (F. varium), which had a unique pattern. Two strains referred by Dr. Elizabeth Sharpe as B. oralis (strains 7CM and J1) differed from each other and from the other strains of B. oralis studied

(NP 333 and WPH 61). Three clinical isolates (GNAB 45, GNAB 46 and GNAB 47) gave bizarre results; one strain did not grow well (WPH 80); three strains did not fit the pattern for a subspecies but could be identified as B. melaninogenicus sp., and one strain resembled B. corrodens.

One strain referred as B. oralis (strain 30) produced brown-pigmented colonies on blood agar and was identified as B. melaninogenicus ss. melaninogenicus. Pigment production developed slowly on blood agar but more rapidly when lysed blood was incorporated in the medium. It was also observed that strains of B. melaninogenicus ss. melaninogenicus produced colonies which were brown-pigmented rather than black.

The identification scheme (figure (v)) was found to be useful in identifying these 53 strains, and most of the strains that did not fit into the scheme were in areas in which it was appreciated further study was needed. As part of a study of possible improved methods of isolating Gram-negative, non-sporing anaerobes from the mouth a number of strains were isolated (see Appendix Ic) which were identified by the scheme given in figure (v). Of the 37 strains of B. melaninogenicus listed in Table 25, B. melaninogenicus ss. intermedius and ss. asaccharolyticus could be identified. Of the ten strains of B. melaninogenicus ss. melaninogenicus that did not produce a pattern of results fitting with those in figure (v), four strains failed to liquefy gelatin and six were resistant to the penicillin disk. These findings must be accepted as variations within the B. melaninogenicus

ss. melaninogenicus group. Resistance of some strains of B. melaninogenicus to penicillin has also been noted by Sutter, Vargo and Finegold (1975).

Of the 30 isolates listed in Table 26, 12 were B. melaninogenicus. Six strains of B. melaninogenicus ss. intermedius and one strain of B. melaninogenicus ss. asaccharolyticus were identified according to the scheme given in figure (v). The occurrence of B. melaninogenicus ss. asaccharolyticus in the mouth is an interesting finding as this strain is usually regarded as a commensal of the intestine and vagina. A few strains of this subspecies have been isolated in this work but they have usually been associated with severe periodontal disease or isolated from abscesses. Strains of B. melaninogenicus ss. melaninogenicus which were penicillin resistant or did not liquefy gelatin were again recovered and this confirms the finding made earlier with the strains listed in Table 25.

The most interesting strains in these isolates were the five strains identified as Bacteroides species. These organisms resembled B. melaninogenicus ss. melaninogenicus except in their failure to produce brown pigment. The pattern of results obtained with these five strains also resembles that obtained for B. ochraceus except that the Bacteroides species are strict anaerobes. These organisms may form a new un-named group or may be B. oralis variants that do not ferment rhamnose and which fail to grow in Victoria blue 4R.

Report to the
International Committee of Systematic Bacteriology

The work presented in this study formed part of an investigation of Gram-negative, non-sporing anaerobes conducted by the Microbial Pathogenicity Research Laboratory in the Department of Bacteriology of the University of Edinburgh. Part of this investigation involved an examination of 12 strains of B. oralis, B. ochraceus and B. melaninogenicus ss. melaninogenicus referred to the laboratory by the International Committee of Systematic Bacteriology, Taxonomic Sub-committee on Gram-negative Anaerobic Rods.

These 12 strains together with six referred by other interested workers and 22 strains isolated from subgingival dental plaque were studied in the full range of tests described in Section A of the Materials and Methods chapter. Analysis of the acid end-products of glucose fermentation was performed by Mr. Arthur Deacon. The report submitted to the I.C.S.B. Taxonomic Sub-committee on Gram-negative Anaerobic Rods is included as Appendix IV (Tables appertaining to this report are placed in the pocket inside the back cover). From the studies carried out on these strains a number of characteristics were noted which relate to other work presented here.

1) B. melaninogenicus ss. melaninogenicus strains were strict anaerobes that produced brown colonies on media containing blood. Strains were inhibited by the bile salts and dyes used in the tests. In general these organisms were sensitive to neomycin,

rifampicin and metronidazole and most strains were sensitive to penicillin in the specified disk tests. The results of tests for hydrolysis of aesculin and dextran, gelatin liquefaction, lipase production and resistance to colistin varied between strains and no apparent relationship between results of these tests was noted.

2) B. oralis strains were also strict anaerobes which failed to produce obvious pigment although a pale-brown colour was noted in colonies grown on lysed-blood agar for seven days. Some strains were indistinguishable from B. melaninogenicus ss. melaninogenicus except for the production of pigment whereas others were able to grow in Victoria blue 4R and to ferment rhamnose. Gas-liquid chromatographic analysis of the acid end-products of glucose fermentation did not assist in the separation of B. oralis from B. melaninogenicus ss. melaninogenicus strains (Mr. Arthur Deacon, personal communication). The similarity of the analyses of acid end-products for these two groups may explain why two strains of B. melaninogenicus (ATCC 15930 and 30), which produce a distinct pigment, have previously been classified as B. oralis by other workers. There does remain, however, an indication that black-pigmentation may not be a satisfactory method of differentiating organisms presently identified as B. melaninogenicus ss. melaninogenicus and B. oralis. Indeed for some strains this is the only characteristic in which members of these two groups differ and the pigment produced by some strains is pale brown and much less definite than the black or dark brown pigment characteristic of B. melaninogenicus strains belonging to the

other two subspecies. This view is supported by Sundqvist (1976) who reported similar problems with faint-pigment-producing colonies.

3) B. ochraceus strains were able to grow in air plus CO₂. They gave similar results to those obtained with B. melaninogenicus ss. melaninogenicus strains in fermentation and other biochemical tests except that they did not digest gelatin. All strains tested hydrolysed dextran. The strains were further distinguished by being resistant to metronidazole and colistin.

Methods of isolating

Gram-negative non-sporing anaerobes with special reference to their isolation from the mouth

Problems of growth of a number of strains have been noted in the initial study of 165 organisms listed in Appendix Ia. The groups "Mxii" in Table 14 and "R" in Table 16 indicate some of the strains that did not grow in all the media used in these tests. In the preliminary survey of clinical sampling methods, further problems of growth were encountered especially when pure subcultures were attempted. The method of plating such exacting strains shown in figure (ii) was an attempt to isolate organisms and lyophilise them for future use in experiments to produce improved growth media.

Strains lost from clinical material could have died in

transit from desiccation or lack of nutrients or exposure to oxygen. They could fail to grow on solid or in liquid media if these media did not contain the necessary range of nutrients. The samples would always contain less demanding organisms which could probably grow more rapidly than the Gram-negative, non-sporing anaerobes and might grow in competition with strains that grow more slowly. Antibacterial substances may be produced by some strains and these could inhibit the growth of the Gram-negative, non-sporing anaerobes.

It was thus necessary to investigate a number of aspects of the sampling procedure that might contribute to the loss of organisms apparent with the methods adopted in the preliminary survey of clinical sampling.

A number of elaborate techniques have been reported for the anaerobic collection and transport of specimens to the laboratory in studies in which further handling was done in an anaerobic cabinet. Tubes previously filled with oxygen-free gas have been advocated for the collection of specimens (Sutter et al., 1972; Rosenblatt, Fallon and Finegold, 1973). For samples removed from the oral cavity, oxygen-free gas has been passed over the sampling site and the sample removed in this stream of gas and used to inoculate roll-tubes (Berg and Nord, 1973). Sutter et al. (1972) recommended the use of a portable, positive-pressure, low-oxygen box for the collection of anaerobic specimens required for quantitation.

The collection of specimens from sites contaminated with normal commensal flora presents a problem to the clinical microbiologist (Bartlett, 1974). As many Gram-negative anaerobic bacilli associated with infection are also commensal in some site in the body, the problem of recognising true association with disease is obvious. For this reason a number of modifications of clinical sampling procedures have been advocated. Moore and Russell (1972) found a great increase in the number of pure cultures obtained from the pus of dental abscesses when pus was aspirated rather than collected on a swab after the abscess had been incised. Other surgical approaches for sample collection include transtracheal aspiration for the collection of sputum, and urine collection by supra-pubic aspiration from the bladder (Bartlett, 1974). Frederick and Braude (1974) recommend a Caldwell-Luc approach to the maxillary antrum for collection of specimens for anaerobic bacteriology. Endodontic specimens also require special precautions especially if the root canal is intact (Møller, 1966; Fulghum, Wiggins and Mullaney, 1973; Berg and Nord, 1973; Kantz and Henry, 1974; Sundqvist, 1976).

The collection of specimens from the gingival crevice is more difficult if contamination from epithelium, supragingival plaque and saliva is to be avoided. Swabs are unsuitable for this site because of their size. Cowley and Egelberg (1963) recommended the use of a steel blade 0.05 mm thick which can be inserted carefully into the crevice; the instrument is then placed in broth and the sample dispersed. Kelstrup (1966a)

covered the superficial structures with an iodine varnish which was pierced by the sampling device, a platinum loop. Henry, Kantz and Braden (1974) have used a sterile micropipette in this site. One important aspect of sampling the often glutinous material of dental plaque or subgingival debris is the ease with which this small sample falls off smooth metal sampling instruments especially if the specimen is moistened with saliva or gingival fluid. For this reason an abrasive strip was used by Bowden, Hardie and Slack (1975).

In the present study no attempt was made to avoid slight contamination of the sampling instrument by saliva or mucosal contact. The aim of the sampling procedure was to find the most effective method of isolating oral bacteroides in the laboratory and it was thought unlikely that all specimens submitted to a laboratory could reliably be free of some contamination from the gingival margin or supragingival plaque. Thus the isolation of the organism, its subsequent growth in pure culture and its later identification formed the chief purpose of the sampling procedure. The chisel-edged wooden sticks used for collecting samples allowed some adherence of the specimen to the sampling device. The sticks could be easily sterilised by autoclaving in test-tubes, were easy to prepare, and were inexpensive. Furthermore, short sticks could be placed in the transport and enrichment media at the chairside, and longer sticks were useful for the direct plating of the specimen at the chairside.

Although a number of workers have advocated the use of apparatus for gassing samples with oxygen-free gas from the moment of the removal from the mouth, or even prior to this, (Gordon, Stutman and Loesche, 1971; Berg and Nord, 1973) it has also been demonstrated that bacteroides organisms are less oxygen sensitive than hitherto thought and can survive for some considerable time in the aerobic conditions of a room (Loesche, 1969; Tally et al., 1975). No special gassing procedures were therefore adopted in this study. It has been noted by Sutter et al. (1972) that some oral organisms may be encountered which are more oxygen sensitive; for these, special procedures may be required. No indication of the identity of these exacting strains was given. The various aspects of the clinical sampling procedure which were investigated in detail are discussed below.

Studies on transport media

The development of a suitable transport medium for oral specimens by Möller (1966) was a considerable advance. Direct plating of the specimen is not always feasible in the clinic and is wasteful if media are to be used freshly prepared as recommended by Watt (1972a). Yrios et al. (1975) found that desiccation presented the greatest threat to anaerobic organisms in small samples even when the atmosphere was oxygen-free. Two transport media have been suggested for oral bacteria, reduced Transport Fluid (Syed and Loesche, 1972) and VMG II (Möller, 1966). Thioglycollate without dextrose or indicator and Gordon's diluent (Gordon, Stutman and Loesche, 1971) were used as control media in

the experiment comparing transport media. Transport media should only be used for protection of organisms in the specimen during transfer from the clinic to the laboratory and once in the laboratory the sample should be treated promptly. In the present survey the delay in plating out the specimen was only $1\frac{1}{2}$ h and it is likely that any of the media used would have given satisfactory results. The recovery of the two strains of B. melaninogenicus in VMG II was, however, considerably better than with the other media and so it seemed appropriate to use this transport medium in the clinic. The increase in numbers of organisms recovered in all media except Gordon's diluent at about 2 h is interesting and needs further investigation. It may be caused by the completion of division of organisms which were undergoing mitosis at the time of inoculation of the medium. The continued rise in numbers of B. melaninogenicus ss. intermedius NCTC 9338 in VMG II and Reduced Transport Fluid may be an artefact or may indicate that this strain can grow to a limited degree in these media. Although it would appear from these pilot experiments that the test organisms survived well in VMG II, an evaluation of this transport medium and Reduced Transport Fluid, involving the quantitative recovery of exacting organisms from clinical specimens, is required. Syed and Loesche (1972) noted a better recovery of plaque bacteria from Reduced Transport Fluid but at time intervals up to 24 h the recovery of these organisms in VMG II and Reduced Transport Fluid was similar.

Studies in liquid media

Cooked-meat broth (see Appendix II) was routinely used

in this laboratory. Forgan-Smith and Darrell (1974) found that USP thioglycollate (Oxoid CM 173) gave the best rapid recovery of Gram-negative anaerobes from blood but that cooked-meat medium prepared in their laboratory gave a longer survival of these organisms. Loesche, Socransky and Gibbons (1964) had used a thioglycollate medium as the basis of their Bacteroides Maintenance Medium (see Appendix II). Other workers had used media containing trypticase such as Sweet E (Holdeman and Moore, 1973); and B.M. broth (Williams et al., 1975). Our modification of B.M. broth takes account of the work of Lev, Keudell and Milford (1971) in the use of sodium succinate as a growth factor for B. melaninogenicus. Serum was also omitted from our medium because the small amounts of carbohydrate in serum were found to affect the fermentation tests in an unpredictable manner and for this reason it was thought that serum-free media would give a more standardised test. The modified medium E and modified B.M. broth allowed good growth of the test strains; as the modified B.M. broth was more easily prepared it was used as the basic liquid medium for the clinical sampling.

Studies on solid media

Blood agar has been used by most workers for the recovery of B. melaninogenicus which is so readily identified by its black-pigmented colonies when grown on this medium. The addition of menadione to this medium was advocated by Gibbons and Macdonald (1960) and Dowell, Hill and Altemeir (1962). Finegold, Sugihara and Sutter (1971) suggested the use of lysed blood to

speed up the production of black-pigment in the colonies, a point that the present studies confirm (see plates IX and X). Columbia agar base (Oxoid) was found to give better recovery of anaerobes than Oxoid Blood Agar Base No. 2 (Dr. Brian Watt, personal communication) and was therefore used in the solid media employed in this study. In a comparison of four plating media, Hunt, Sandham and Gilmore (1969) found Brain-Heart-Infusion agar gave the best recovery of organisms from dental plaque. Syed and Loesche (1973) reported that a modification of the medium 10 of Caldwell and Bryant (1966), which they termed "MM10-sucrose-blood agar", gave the best recovery of oral organisms from dental plaque. The results of the experiments in this study support the findings of Syed and Loesche (1973) that modified medium 10-sucrose-blood agar gives a good recovery of anaerobes. A problem with this medium, however, is the failure of B. melaninogenicus strains to produce black-pigmented colonies and this is a drawback to the more rapid identification of this organism. The production of black-pigmented colonies on modified B.M.-blood agar gave this medium a considerable advantage over modified medium 10-sucrose-blood agar.

Although *Fusobacterium* species have been found to grow well on most media that support the growth of *Bacteroides* species a number of media were initially developed for fusobacteria. Kasei (1955) found a tryptone, starch, yeast extract and dextrose combination useful. Omata and Disraely (1956) developed a medium containing casitone, yeast extract, glucose, NaCl and L-cysteine HCl and this has also been used as the basic medium for a selective

medium for fusobacteria. More recently a medium has been recommended by the American Type Culture Collection (1972) for Leptotrichia buccalis which contains nutrient broth, yeast extract, glucose, cysteine and haemin.

Studies on selective media

Gram-negative, non-sporing anaerobes occurring in infection or as part of the normal flora are nearly always accompanied by other less demanding organisms. Selective agents have thus been found useful for the isolation of Gram-negative, non-sporing anaerobes. Early studies on the isolation of these organisms employed dyes such as crystal violet (Omata and Disraely, 1956; Onisi, 1959) or ethyl violet (Baird-Parker, 1957), and some dyes are still recommended such as China blue (van Palenstein-Helderman and Winkler, 1975). One problem with most dyes is that many will permit the growth of Fusobacterium species and Leptotrichia species. Some will allow the growth of B. fragilis, but B. melaninogenicus is inhibited by all dyes so far examined.

Antibiotics were also useful and were incorporated with dyes in the selective media developed for fusobacteria by Omata and Disraely (1956) and Baird-Parker (1957). Finegold, Miller and Posnick (1965) found that the resistance of Gram-negative anaerobes to aminoglycosides could be used to aid their isolation. Kanamycin and vancomycin proved suitable for B. fragilis, B. funduliformis and B. melaninogenicus. Paromycin and vancomycin were suitable for the isolation of fusobacteria. This approach

was consolidated in a chapter in Isolation of Anaerobes (Finegold, Sugihara and Sutter, 1971) and was the method adopted by Zabransky (1970) in a survey of the isolation of anaerobes from clinical specimens. Kanamycin was useful in the isolation of B. melaninogenicus from the mouth (Loesche, Hockett and Syed, 1971; Okuda, Nakamura and Yonezawa, 1974).

The selection of certain Fusobacterium species from Bacteroides species can be achieved by incorporating rifampicin in the medium (Sutter, Sugihara and Finegold, 1971). A number of strains of fusobacteria are, however, sensitive to rifampicin (see Tables 17a and 17b). McCarthy and Snyder (1963) stated that vancomycin and streptomycin were useful agents in selecting fusobacteria from bacteroides.

Phenylethyl alcohol has proved useful in selecting many anaerobes including bacteroides and fusobacteria whilst inhibiting coliform organisms (Ninomiya et al., 1970). The incorporation of crystal violet into this medium made it more selective for F. necrophorum (Fales and Teresa, 1972).

In the present study, kanamycin and vancomycin were used as the selective agents and most strains of Gram-negative, non-sporing anaerobes grew as well in this medium as on the control medium. Some inhibition of growth was noted with strains of B. melaninogenicus ss. melaninogenicus and ss. asaccharolyticus when vancomycin was incorporated into the basic medium. This was largely overcome by the inclusion of 0.25% sodium succinate

into the basal medium of modified B.M.-blood agar. The efficacy of this medium in selecting B. melaninogenicus strains is discussed in the section on the clinical sampling survey but it can be noted clearly in plates XV and XVI.

Studies on enrichment media

These media were developed as an attempt to allow B. melaninogenicus to grow in preference to other organisms and so allow the presence of B. melaninogenicus to become apparent in a sample even when it was present as a low proportion of the total number of organisms. The medium consisted of kanamycin and vancomycin as selective agents in the modified B.M. broth found to be most satisfactory for the growth of bacteroides organisms. The value of this medium was assessed in the clinical sampling survey (q.v.) and the initial experiments were merely to determine that adequate growth of test strains occurred and that common commensal strains were inhibited.

The clinical sampling survey

The evaluation of the test media was first based on analysis of their efficiency in the isolation of B. melaninogenicus by the various test routes. B. melaninogenicus was chosen as an indicator organism because it produced a characteristic black colony which allows it to be readily identified. This species is also quite demanding and provides a good presumptive index of acceptable or unsuitable methods; if a less exacting B. fragilis strain had been chosen as an indicator, the difference might have been less clear.

The results clearly indicate the value of using a selective medium containing both kanamycin and vancomycin. Samples grown on this selective medium gave a good recovery of B. melaninogenicus organisms from the 50 patients and this recovery was not dependent on the initial method of treatment of the sample. The absence of correlation between the degree of gingival or periodontal disease with the success of isolating a strain of B. melaninogenicus indicates that the sampling and culture technique is suitable for sampling even from healthy mouths with little gingival debris.

No clear indication emerged from the statistical analysis of the data as to the best method of treating the sample. A subjective impression was gained that the use of the transport medium involved a dilution of the sample which gave discrete colonies after incubation. These colonies were more easily

subcultured than those observed on the directly seeded plates in which a profuse growth was often observed. The use of an enrichment broth did not improve the isolation rate and was a time-consuming procedure in which samples were incubated for 24 h or 48 h longer than those processed via the transport medium or those directly seeded on to plates. As the survey showed that B. melaninogenicus strains could be recovered even from healthy mouths where there was little gingival debris there is no need for an enrichment procedure to allow small numbers of B. melaninogenicus to grow preferentially before inoculation of a plate. It was not thought necessary to inoculate all three solid media with samples of the enrichment broth cultures as any organisms viable after 48 h in the enrichment broth, which contains kanamycin and vancomycin, would be likely to be resistant to these antibiotics. No improvement in recovery of B. melaninogenicus on non-selective media was observed after incubation of the enrichment broth had been extended up to 48 h.

If a quantitative study were to be undertaken it is not clear from these results whether results would differ when selective media or a non-selective medium were used. Samples should probably be divided and used to inoculate both selective and non-selective media. The quantitative recovery of many fusobacteria would only be possible on non-selective media because they are somewhat sensitive to kanamycin. A full statistical analysis of these data has not yet been possible. This analysis should be carried out on the whole sample with each variable examined for each patient to reduce inter-patient variation

(Mrs. Margaret Shotter, personal communication). A computer programme has not yet been developed in Edinburgh which is capable of assimilating the data for such an analysis. The results as presented do indicate the advantages of certain isolation procedures and are sufficient for the present study, but they have generated considerable interest in the Department of Statistics of this University and further research into the analysis of these data is being undertaken by that Department.

It is clear from the results of the isolation procedure that a number of organisms isolated in mixed culture failed to grow on subculture. Similarly it may be assumed that a number of organisms present in the sample did not grow even in mixed culture, although no quantitative studies have been performed to illustrate that point in this particular study. When a sample is removed from the mouth the exposure of the specimen to oxygen or to desiccation may cause a steady decrease in the number of viable organisms that can be recovered. Other factors will also affect the recovery of organisms from the specimen such as the lack of nutrients in the culture medium; competition from more rapidly growing strains and bacterial antagonism. These factors may have a more profound effect on organisms that are already damaged. Moderation of some of these factors may well partially compensate for the adverse effects of others. The contribution of any single factor to the loss of organisms is thus difficult to assess. It is often assumed that exacting strains are more sensitive to exposure to oxygen than less exacting strains, but in this study the improved media adopted

for the later clinical sampling surveys gave a marked improvement in recovery of B. melaninogenicus, which is widely regarded as a demanding organism. A future study of the inter-relationship of nutrition and sensitivity of these exacting strains to oxygen would be a valuable contribution to the understanding of their growth and survival.

In the second experiment to evaluate the test media in the clinical situation, attempts were made to isolate as many different types of Gram-negative non-sporing anaerobes as possible from the mouth. The sample of subjects was small but the amount of work was considerable; this experiment served to show whether or not the media found useful for the isolation of B. melaninogenicus were likely to allow the isolation of other Gram-negative anaerobes that are known to inhabit the oral cavity. It was clear from the range of organisms isolated that the use of VMG II transport medium and the selective medium containing kanamycin and vancomycin did allow a wide range of these organisms to be isolated (see Table 26).

B. fragilis was not isolated from any of the specimens and this supports the conclusion of numerous other workers that this species is not an oral commensal. Very few strains of B. melaninogenicus ss. melaninogenicus have previously been reported; Williams et al. (1975) used only one strain and Holdeman and Moore (1973) based their characterisation in the Anaerobe Laboratory Manual on studies with two strains. Five strains were isolated from the five samples in the present study and this subspecies was also detected in 42 of the 50 samples collected in

the clinical sampling survey. Failure to recognise

B. melaninogenicus ss. melaninogenicus may be explained by the atypical colonial morphology of the strain on media such as modified B.M.-blood agar (see colony "B" on plate XVI).

B. melaninogenicus ss. intermedius has been reported as being the subspecies most commonly encountered in the mouth (Williams et al., 1975) and indeed this subspecies was found in all five samples.

B. melaninogenicus ss. saccharolyticus is most frequently isolated from faeces, vaginal swabs and abdominal wounds. It is rarely isolated from the mouth (Williams et al., 1975; Dr. J.M. Hardie, personal communication) and only one strain was isolated from the five samples in this survey. Out of the complete series of strains studied, all nine strains of B. melaninogenicus ss. melaninogenicus that were isolated from the mouth were sampled from gingival crevices with severe periodontitis or were from infected oral wounds.

The isolation technique involving the use of VMG II transport medium and inoculation of modified B.M.-blood agar with kanamycin and vancomycin allowed the isolation of all the recognised species and subspecies of oral, Gram-negative, non-sporing anaerobic bacilli. In addition a number of strains were found that did not fit into any of the recognised species and this indicates that the method may be useful for further studies on oral, Gram-negative, non-sporing anaerobes in which the occurrence of these strains and their identity may be more fully investigated.

The study has shown that relatively simple conventional

laboratory techniques can be adapted for use with these often exacting organisms. The methods of sampling, transport, culture and identification of Gram-negative, non-sporing, anaerobic bacilli described here could be adopted by most routine clinical laboratories. Furthermore the techniques have proved useful at the research level and should enable an increased knowledge of the presence of these organisms as part of the commensal flora and an awareness of their role in disease.

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APPENDICES

APPENDIX I

Strains used in these studies:

- Ia Sources of the 165 strains used for the development of the scheme for the identification of Gram-negative, non-sporing anaerobic bacilli.
- Ib Sources of additional strains on which all tests were performed.
- Ic Sources of strains identified in the shortened series of tests used as an identification scheme.

APPENDIX Ia

Sources of the 165 strains used for the development of
the scheme for the identification of
Gram-negative non-sporing anaerobic bacilli

| Strain | Source |
|--|---|
| <u>Bacteroides fragilis ss. fragilis</u>) NCTC 8560, 9343, 9344, 10581,) 10584.) | National Collection of Type Cultures (N.C.T.C.), Central Public Health Laboratory, Colindale Avenue, London. |
| <u>B. fragilis ss. thetaiotaomicron</u> , NCTC 10582 ATCC 8492 | N.C.T.C. Dr. Ella M. Barnes, A.R.C. Food Research Institute, Colney Lane, Norwich. |
| <u>B. fragilis ss. vulgatus</u> NCTC 10583 ATCC 8482 | N.C.T.C. Dr. Ella M. Barnes |
| <u>B. fragilis ss. ovatus</u> ATCC 8483 | Dr. Ella M. Barnes |
| <u>B. fragilis ss. distasonis</u> ATCC 8503 | Dr. Ella M. Barnes |
| <u>B. melaninogenicus ss. intermedius</u> NCTC 9336, 9338 | N.C.T.C. |
| <u>B. melaninogenicus ss.</u> <u>asaccharolyticus</u> NCTC 9337 | N.C.T.C. |
| <u>B. melaninogenicus ss.</u> <u>melaninogenicus</u> VPI 4196 | Virginia Polytechnic Institute, Anaerobe Laboratory, Blacksburg, Va 24060, USA. |
| <u>B. oralis</u> ATCC 15930 (now <u>B. melaninogenicus</u> - Holbrook & Duerden, 1974) | American Type Culture Collection (A.T.C.C.) 12301 Parklawn Drive, Rockville, Md 20852, USA. |
| <u>B. oralis</u> NP333 | Dr. G.H. Bowden, London Hospital Medical College |
| <u>B. ochraceus</u> WPI 2845 | Virginia Polytechnic Institute |

APPENDIX Ia (continued)

| | |
|--|--|
| <u>B. necrophorus</u> (<u>Fusobacterium</u>) <u>necrophorum</u>) NCTC 10575, 10576,) 10577) | N.C.T.C. |
| <u>Fusobacterium necrogenes</u> NCTC 10723 | N.C.T.C. |
| <u>F. polymorphum</u> NCTC 10562 | N.C.T.C. |
| <u>Leptotrichia buccalis</u> NCTC 10249 | N.C.T.C. |
| <u>L. dentium</u> NCTC 10206 | N.C.T.C. |
| <u>B. necrophorus</u> (? <u>Clostridium</u> sp) NCTC 7155 (see Barnes et al., 1968) | N.C.T.C. |
| Anaerobic "corroding bacilli" Nos. 124, NC-1, NC-2, NCL 20) | Dr. A.L. James, Newcastle- upon-Tyne Polytechnic |
| <u>B. melaninogenicus</u> Nos. 2296,) 3502, 3586) | Dr. R. Wiseman, Bangour General Hospital, Broxburn, West Lothian |
| Routine clinical laboratory) isolates - 89 strains) | Royal Infirmary, Edinburgh Western General Hospital, Edinburgh |
| Gingival crevice material -) 17 strains) | Edinburgh Dental Hospital |
| Normal, human faeces -) 26 strains) | Bacteriology Department, University of Edinburgh |

APPENDIX Ib

Sources of additional strains on which
all tests were performed

| Strain | Source |
|---|--|
| <u>Sphaerophorous varians</u> NCTC 10560 | N.C.T.C. |
| <u>Bacteroides corrodens</u> nos. 151/ RV, RV/ 2, 143A | Dr. A.L. James, Newcastle- upon-Tyne Polytechnic |
| <u>Bacteroides oralis</u> nos. J1, 7CM, 30 | Dr. Elizabeth M. Sharpe, National Institute for Research in Dairying, Reading |
| <u>Bacteroides melaninogenicus</u> nos. G11a-d, P11a-k, AB13a-f, UJB13a-c, D13a-f | Prof. Göran Sundqvist, Dept. of Endodontics, University of Umeå, Sweden |
| <u>Bacteroides</u> sp. no. B20-a (found to be aerobic) | Prof. Göran Sundqvist |
| Routine clinical laboratory isolates - 11 strains | Royal Infirmary, Edinburgh, Western General Hospital, Edinburgh |
| Gingival crevice material - 29 strains | Edinburgh Dental Hospital |

APPENDIX IcSources of strains identified in the shortened series of tests
used as an identification scheme

| Strain | Source |
|---|---|
| <u>Bacteroides melaninogenicus</u> VPI 9169 | Dr. J.M. Hardie, London Hospital Medical College, Dental School, Turner Street, London |
| Routine clinical laboratory isolates - 4 strains | Royal Infirmary, Edinburgh, Western General Hospital, Edinburgh |
| Gingival crevice material - 67 strains | Edinburgh Dental Hospital |

APPENDIX II

Methods of preparing the media described in

Materials and Methods Section B

APPENDIX IIMedia used in experiments in Section BB.M. Broth (Williams et al., 1975)

| | |
|--|-----------|
| Trypticase | 1% |
| Proteose peptone (Oxoid) | 1% |
| Yeast extract (Difco) | 0.5% |
| Sodium chloride | 0.5% |
| Adjust pH to 7.4, autoclave at 121°C for 15 min and to cooled medium add filter-sterilised solutions of: | |
| Haemin | 5 µg/ml |
| Menadione | 1 µg/ml |
| L-cysteine HCl | 0.075% |
| Horse serum (Wellcome) | 2% |
| 10% sodium carbonate | few drops |

B.M. Enrichment Broth

| | |
|--------------------------|-------|
| Trypticase (BBL) | 1% |
| Proteose peptone (Oxoid) | 1% |
| Yeast extract (Difco) | 0.5% |
| Sodium chloride | 0.5% |
| Sodium succinate | 0.25% |

Dissolve in distilled water, adjust pH to 7.4. Add 20 ml volumes to approximately 1 cm depth cooked-meat particles in 1 oz McCartney bottles. Autoclave and store at 4°C.

To cooled medium add:

| | |
|-----------|---------|
| Haemin | 5 µg/ml |
| Menadione | 1 µg/ml |

| | |
|----------------|-----------|
| L-cysteine HCl | 0.075% |
| Kanamycin | 75 µg/ml |
| Vancomycin | 2.5 µg/ml |

Store anaerobically at room temperature overnight a) as a sterility check and b) to reduce the medium. Tighten caps on removal from the jar.

Brain-Heart-Infusion Broth

| | |
|------------------------------------|-------|
| Brain-heart-infusion broth (Oxoid) | 3.7 g |
|------------------------------------|-------|

Dissolve in 100 ml distilled water, dispense in 10 ml amounts into test-tubes and autoclave. Store at 4°C. Before use steam for 30 min, cool rapidly and add:

| | |
|-----------|---------|
| Haemin | 5 µg/ml |
| Menadione | 1 µg/ml |

Cooked-Meat Broth (modified from Cruickshank, 1968)

Boil the minced ox-heart and dry. Dispense into test-tubes to a depth of approximately 2 cm.

| | |
|------------------------------|-------|
| Nutrient broth no. 2 (Oxoid) | 2.5 g |
|------------------------------|-------|

Dissolve broth in 100 ml distilled water and add 10 ml to each test-tube. Autoclave and store at 4°C. Steam for 30 min and cool rapidly immediately before use.

Gordon's Diluent (Gordon, Stutman and Loesche, 1971)

| | |
|--------------------------------|--------|
| 0.067M Phosphate buffer pH 7.2 | |
| Peptone (Difco) | 0.1% |
| Sodium thioglycollate | 0.001% |
| Resazurin | 0.001% |

Dispense in 5 ml amounts in bijoux bottles and autoclave. Store

anaerobically at 4°C with tightened stoppers. Loosen stoppers and hold overnight, anaerobically, at room temperature before use.

Haemin Solution

| | |
|--------------|-------|
| Haemin (BDH) | 50 mg |
| N/10 NaOH | 10 ml |

Dissolve haemin in NaOH and add 90 ml distilled water; membrane filter and store at 4°C.

Horse-Digest Broth

| | |
|---|--------|
| Best quality steak, defatted and minced | 1.8 kg |
| Tap water | 3 l |

Heat to 80°C in a steamer, stirring constantly. Add 4 l cold tap water and 25 g anhydrous sodium carbonate and adjust pH to 8.0.

| | |
|------------|------|
| Pancreatin | 35 g |
|------------|------|

Add pancreatin and hold in oven at 60°C for 6 h to digest meat.

Add 40 ml HCl (pure) and steam for 30 min to stop digestion. Leave overnight at 4°C to allow fat to rise. Siphon off supernate and filter. Add 70 g proteose peptone (Difco) and adjust pH to 8.0. Add anhydrous calcium chloride (8.78 g), steam to dissolve, cool, filter and adjust pH to 7.4-7.6.

Dispense in 10 ml amounts in test-tubes. Autoclave and store at 4°C. Steam for 30 min and cool rapidly before use.

Lysed-Blood Agar with Menadione

To sterile, molten, Columbia agar (Oxoid) at about 50°C add 5% saponin-lysed, human blood (Cruickshank, 1968) and 1 µg/ml menadione.

Menadione Solution (Barnes and Impey, 1971)

Dissolve 10 mg menadione (Sigma) in 1 ml ethyl alcohol. Add distilled water to 100 ml. Membrane filter and store at 4°C in dark bottles.

Modified B.M. Broth (modified from Williams et al., 1975)

| | |
|-----------------------|-------|
| Trypticase (BBL) | 1% |
| Proteose peptone | 1% |
| Yeast extract (Difco) | 0.5% |
| Sodium chloride | 0.5% |
| Sodium succinate | 0.25% |
| Ionagar no. 2 (Oxoid) | 0.1% |

Steam to dissolve and adjust pH to 7.4. Tube off in 10 ml volumes. Autoclave and store at 4°C. For use steam for 30 min and cool rapidly to 37°C. Add:

| | |
|----------------|---------|
| Haemin | 5 µg/ml |
| Menadione | 1 µg/ml |
| L-cysteine HCl | 0.075% |

Modified B.M.-Blood Agar

| | |
|-----------------------|-------|
| Trypticase (BBL) | 1% |
| Proteose peptone | 1% |
| Yeast extract (Difco) | 0.5% |
| Sodium chloride | 0.5% |
| Sodium succinate | 0.25% |
| Ionagar no. 2 (Oxoid) | 1.2% |

Dissolve ingredients in distilled water and adjust pH to 7.4.

Autoclave and cool to approximately 50°C and add:

| | |
|---------------------------|---------|
| Saponin-lysed human blood | 5% |
| Menadione | 1 µg/ml |
| L-cysteine HCl | 0.075% |

Modified Medium E (Mr. Arthur Deacon, personal communication)

Buffered salt solution A (pH 7.0)

| | |
|---|--------|
| KH_2PO_4 | 0.69 g |
| K_2HPO_4 | 0.85 g |
| CaCl_2 (anhydrous) | 10 mg |
| $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ | 10 mg |
| NaCl | 0.1 g |
| Distilled water | 100 ml |

Place the dry chemicals in a flask and then add the water.

Solution B

| | |
|----------------------------|-------------|
| Pyruvic acid | 1 ml/l |
| Biotin (50 µg/ml solution) | 5 µg/100 ml |
| L-cysteine HCl | 0.075% |
| Glutamine | 5 mg% |
| Distilled water | 17 ml |

Weigh the glutamine, add the L-cysteine HCl and cool to 4°C.

Add pyruvic acid and biotin solution to distilled water and cool to 4°C, mix, membrane filter and store frozen.

Mixture C

| | |
|-----------------------|-------|
| Gelatin (Difco) | 0.3 g |
| Trypticase (BBL) | 1.0 g |
| Yeast extract (Difco) | 0.3 g |
| Sodium thioglycollate | 50 mg |

| | |
|--------------------------|--------|
| Ionagar no. 2 (Oxoid) | 0.1 g |
| Buffered salt solution A | 100 ml |

Steam to dissolve ingredients, check and note pH. Tube off in 10 ml amounts, autoclave and store at 4°C.

For use steam for 30 min, cool rapidly to 37°C and add to Mixture C:

| | |
|-------------------------|---------|
| Inactivated horse serum | 20% |
| Haemin | 5 µg/ml |
| Menadione | 1 µg/ml |
| Solution B | 0.2 ml |

Modified Medium 10-Sucrose-Blood Agar (modified from Syed and Loesche, 1973)

| | |
|--------------------------|---------|
| Trypticase (BBL) | 2 g |
| Yeast extract (Difco) | 0.5 g |
| Haemin | 0.001 g |
| Sodium lactate (60%) | 4 ml |
| Sodium formate | 1 g |
| Potassium nitrate | 0.5 g |
| Mineral solution no. 1* | 38 ml |
| Mineral solution no. 2** | 38 ml |
| Glucose | 1 g |
| Sucrose | 50 g |
| Ionagar no. 2 (Oxoid) | 15 g |
| Distilled water | 874 ml |

Autoclave at 121°C for 20 min. Allow to cool to 50°C and then add:

| | |
|---|------|
| 8% Na ₂ CO ₃ solution (membrane filtered) | 5 ml |
| 0.05% menadione solution (membrane filtered) | 1 ml |

1% dithiothreitol solution 20 ml
 (make 21 ml of 1% soln. dithiothreitol and filter
 just before use)

Defibrinated sheep blood 20 ml

* mineral solution no. 1 contains;

K_2HPO_4 0.6%

** mineral solution no. 2 contains;

NaCl 1.2%

$(NH_4)_2SO_4$ 1.2%

$CaCl_2$ 0.12%

NCTC 109 TC Medium (Difco)

Dehydrated NCTC 109 TC Medium (Difco) 0.97 g

Distilled water 98 ml

Dissolve by slowly adding the medium to the water while stirring.

Add 2 ml of 10% $NaHCO_3$ solution, and then add 5 ml inactivated horse serum. Sterilise by membrane filtration and tube off aseptically in 10 ml amounts. Store anaerobically at 4°C.

For use remove from anaerobic jar and add to each 10 ml volume;

Haemin 5 µg/ml

Menadione 1 µg/ml

L-glutamine solution* 136 mg/l

* L-glutamine solution made up as 13.6 mg/ml: add 136 mg L-glutamine to 10 ml distilled water at 4°C. When dissolved sterilise by Millipore filter and store frozen at -20°C.

Reduced Transport Fluid (Syed and Loesche, 1972)

| | |
|--------------------------------------|--------|
| Stock mineral solution no. 1* | 75 ml |
| Stock mineral solution no. 2** | 75 ml |
| 0.1M EDTA | 10 ml |
| 8% Na_2CO_3 | 5 ml |
| 1% dithiothreitol (freshly prepared) | 20 ml |
| Resazurin (0.1%) | 1 ml |
| Distilled water | 814 ml |

Sterilise by membrane filtration, dispense in 5 ml amounts in bijoux bottles. Store aerobically at 4°C but incubate anaerobically overnight before use.

* stock mineral solution no. 1 contains:

| | |
|--------------------------|------|
| K_2HPO_4 | 0.6% |
|--------------------------|------|

** stock mineral solution no. 2 contains:

| | |
|------------------------------|-------|
| NaCl | 1.2% |
| $(\text{NH}_4)_2\text{SO}_4$ | 1.2% |
| KH_2PO_4 | 0.6% |
| MgSO_4 | 0.25% |

Selective Media

To molten, modified B.M. medium (qv.) cooled to 50°C after autoclaving add either:

| | |
|-----------------|-----------|
| a) Kanamycin | 75 µg/ml |
| or b) Kanamycin | 75 µg/ml |
| plus Vancomycin | 2.5 µg/ml |

Thioglycollate Medium (modified from Loesche, Socransky and Gibbons, 1964)

| | |
|---|-------|
| Thioglycollate medium without dextrose or indicator (BBL) | 24 g |
| Yeast extract (Oxoid L21) | 0.25% |
| Sodium succinate | 0.25% |
| Distilled water | 1 l |

Dissolve by boiling for 1 min. Dispense into 10 ml amounts and autoclave. Steam this basic medium for 30 min prior to use and cool rapidly to 37°C. Add:

| | |
|-----------|---------|
| Haemin | 5 µg/ml |
| Menadione | 1 µg/ml |

Thioglycollate Medium without Dextrose or Indicator (BBL)
(for transport).

| | |
|---|------|
| Thioglycollate medium without dextrose or indicator (BBL) | 24 g |
| Distilled water | 1 l |

Dissolve the medium, dispense in 5 ml amounts in bijoux bottles and autoclave at 121°C for 20 min. Store aerobically at 4°C but incubate anaerobically at room temperature overnight before use.

VMG II Fluid Storage Medium (Möller, 1966)

Solution A

| | |
|-----------------------|-------|
| Ionagar (Oxoid no. 2) | 10 mg |
| Glass distilled water | 90 ml |

Part B

| | |
|-----------------------|-------|
| Bacto gelatin (Difco) | 1 g |
| Tryptose (Difco) | 50 mg |
| Thiotone (BBL) | 50 mg |

| | |
|--|-------|
| Cysteine HCl (BDH) | 50 mg |
| Mercaptoacetic acid (BDH) (5% solution) | 1 ml |
| Bacteriological charcoal (Oxoid L9) | 1 g |

Salt Solution IIC

| | |
|--|------------|
| Phenylmercuric acetate (BDH) | 30 mg |
| $\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$ (BDH Analar) | 2.4 g |
| KCl (BDH Analar) | 4.2 g |
| NaCl (BDH Analar) | 10 g |
| $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (BDH Analar) | 1 g |
| Sodium glycerophosphate (BDH) | 100 g |
| Glass distilled water | to 1000 ml |

Dissolve the phenylmercuric acetate in about 800 ml glass distilled water by gentle heating. Add the other salts and when dissolved make the volume up to 1000 ml with glass distilled water. Store at room temperature.

To prepare the complete medium:

Dissolve the agar in the glass distilled water by bringing to the boil for about 1 minute. Cool to about 50°C then add the ingredients in part B. Add the required amount of stock solution IIC. Adjust pH to 7.5 with 1N NaOH. Dispense in 6 ml volumes in metal capped bijoux bottles. Autoclave at 121°C for 20 min with the caps loose then tighten the caps immediately on removal from the autoclave. Store at room temperature, aerobically.

APPENDIX III

Names and addresses of suppliers of materials

APPENDIX IIINames and addresses of suppliers of materials

American Type Culture Collection: 12301 Parklawn Drive, Rockville,
Md. 20852, U.S.A.

Baird and Tatlock Ltd.: Romford, Essex RM11HA, England.

BBL Ltd.: York House, Empire Way, Wembley, Middlesex HA9OPS, England.

BDH Chemicals Ltd.: Broom Road, Poole, Dorset BH124NN, England.

British Oxygen Company Ltd.: Special Gases Division, Deer Park Road,
London SW193UF, England.

Difco Laboratories: West Molesey, Surrey, England.

George T. Gurr and Sons Ltd.: P.O. Box 1, Romford, Essex RM11HA,
England.

Hawksley Ltd.: 12 Peter Road, Lancing, Sussex.

Kerrys (Ultrasonics) Ltd.: Hunting Gate, Wilbury Way, Hitchin, Herts.,
England.

Mast Laboratories Ltd.: Liverpool, England.

May and Baker Ltd.: Dagenham, Essex RM107XS, England.

National Collection of Type Cultures: Central Public Health Laboratory,
Colindale Avenue, London, England.

Oxoid Ltd.: Wade Road, Basingstoke, Hampshire RG24OPW, England.

Pharmacia Fine Chemicals AB.: Box 175, S-75104 Uppsala 1, Sweden.

Pye Unicam Ltd.: Cambridge, England.

Sigma London Chemical Co. Ltd.: Norbiton Station Yard, Kingston-upon-
Thames, Surrey KT27BH, England.

Virginia Polytechnic Institute and State University: Anaerobe
Laboratory, Blacksburg, Va. 24060, U.S.A.

APPENDIX IV

Report to the International Committee of Systematic Bacteriology
(I.C.S.B.) Taxonomic Subcommittee on Gram-negative Anaerobic Rods.
(N.B. see tables attached in pocket inside back cover).

REPORT TO THE I.C.S.B. TAXONOMIC
SUBCOMMITTEE ON GRAM-NEGATIVE ANAEROBIC RODS

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Introduction

As part of the collaborative investigation instigated by the Taxonomic Subcommittee on Gram-negative anaerobic rods, 40 strains of Bacteroides melaninogenicus ss. melaninogenicus, B. oralis and B. ochraceus were subjected to a series of tests. These strains included the 12 referred to us by the subcommittee, 6 strains referred by other colleagues and 22 strains isolated from subgingival dental plaque in our laboratory. This work forms part of our continuing studies with B. melaninogenicus and related organisms.

Materials and Methods

Organisms

Bacteroides melaninogenicus ss. melaninogenicus strains WAL 2721* and WAL 2724* (Dr. S.M. Finegold), GUI 1011* and GUI 1034* (Dr. K. Ueno), VPI 4196 (Dr. E. Cato, V.P.I.) and 15 strains isolated from subgingival dental plaque in our laboratory.

Bacteroides oralis strains VPI 7570A* and VPI 5832* (V.P.I., Blacksburg, Va.), J1*, 7CM* and 30* (Dr. E. Sharpe), ATCC 15930* (American Type Culture Collection), NP333 (Dr. J.M. Hardie, London Hospital Medical College), and 7 strains isolated from subgingival dental plaque in our laboratory.

Bacteroides ochraceus (Ristella ochraceus) strains 1956C* and 2467B* (Dr. M. Sebald), VPI 2845 (V.P.I., Blacksburg,

Va.), 10, 79B and 73 (Dr. W.H. van Palenstein-Helderman, Preventive Dentistry Dept., University of Utrecht, Netherlands).

* Strains referred by the I.C.S.B. Taxonomic Subcommittee.

Characterisation of Strains

The 40 test strains were subjected to the following series of morphological, biochemical, tolerance, and antibiotic-disk-resistance tests (for details of media and methods, see Duerden *et al.*, 1976) and to gas-liquid chromatographic (G.L.C.) analysis of their short chain fatty acid metabolic products of glucose.

Morphological and biochemical tests. Microscopic and colonial morphology; haemolytic effect on blood agar; pigment production; motility; lipase activity; oxidase test; catalase test; hydrogen sulphide production; indole production; gelatinase test; aesculin hydrolysis; fermentation of glucose, lactose, sucrose, maltose, rhamnose, trehalose, and mannitol.

Tolerance tests. Growth in the presence of (i) the bile salts sodium taurocholate, sodium deoxycholate, and the combination of sodium taurocholate plus sodium deoxycholate, and (ii) the dyes brilliant green, Victoria blue 4R, gentian violet and ethyl violet.

Antibiotic-disk-resistance tests. Resistance to disks containing neomycin 1000 µg and 10 µg, kanamycin 1000 µg and 30 µg, penicillin 1.5 units, methicillin 10 µg, erythromycin 60 µg, colistin 10 µg, rifampicin 15 µg, lincomycin 2 µg, clindamycin 2 µg,

bacitracin 0.1 unit, vancomycin 15 µg, chloramphenicol 10 µg, tetracycline 10 µg and metronidazole 5 µg.

G.L.C. analysis of short-chain fatty acids

Medium. The strains were grown for 48 h in a glucose-containing medium (PPYG) containing: Proteose peptone (Oxoid) 2%; yeast extract (Difco) 1%; and NaCl 0.5%. Filter-sterilised solutions of the following heat-labile supplements were added aseptically to the (cooled) autoclaved basal medium (pH 7.4) to give final concentrations of: inactivated horse serum, 2%; glucose, 1%; haemin, 5 µg/ml; menadione, 1 µg/ml; cysteine hydrochloride, 0.75%; and Na_2CO_3 , 0.04%. The pH at inoculation was 7.1 ± 0.1 . The inoculum was one drop (0.02 ml) of a 48-h culture in cooked-meat broth.

Chromatograph. A Pye-Unicam series 104 gas chromatograph fitted with heated injection ports and dual flame-ionization detectors was operated isothermally at 190°C with a detector temperature of 250°C. The carrier-gas was oxygen-free nitrogen at a flow-rate of 35 ml/min and the hydrogen flow-rate of both detectors was adjusted for optimum sensitivity. The instrument was fitted with two identical glass columns (1.5 m x 4 mm) containing Chromosorb 101 (Johns-Manville Corp., U.S.A.; supplied by Gas Chromatography Services Ltd., 23 Old Chester Road, Lower Bebington, Wirral, Merseyside, L63 7LA). Columns were packed in the laboratory. The recorder was a Servoscribe 1S model 541.20 (Belmont Instruments, 6 Belmont Drive, Giffnock, Glasgow, G46 7PA) set at the 10 mV range with a recorder speed of 120 mm/h.

Analysis. The procedures used were derived from those of Carlsson (1973).

(a) Volatile acids. Cultures were acidified with 50% sulphuric acid to pH 2.0 and a 0.6 μ l sample of cell-free supernatant was injected directly on to the analysing column without further pre-treatment or extraction. Contamination of the top 2-3 cm of the column occurred during use and required periodic replacement with fresh polymer. The attenuation setting was 2×10^{-2} at the $\times 1$ range setting.

(b) Non-volatile acids. The acidified culture supernates were methylated according to the method of Holdeman and Moore (1972) and a 0.6 μ l sample of the chloroform extract was injected on to the column under the same analysis conditions as for the volatile acids but with an attenuation setting of 5×10^{-2} .

Lactic and succinic acids were detected qualitatively in the analysis of volatile acids, but this was confirmed quantitatively by the methylation procedure.

Standards. Single and combined 0.01 M aqueous standards of the volatile acids were used to establish absolute and relative retention times; 0.02 M standards were used in the analysis of the non-volatile acids. Samples of uninoculated (sterile) PPYG medium were included as controls in every batch of each of the two types of analysis. A PPYG control and a combined acid standard were used to monitor retention times and the sensitivity settings of the instrument as a routine each day.

The approximate concentration values of acids for test

samples were calculated by comparing the corrected peak heights of the test samples with those of the appropriate acid standards.

Results were recorded as follows:

Volatile acids

| | | |
|---------------------|------------------------------|------------|
| Concentration value | > 10 $\mu\text{M/ml}$: | ++ |
| " " | 1.1 - 10 $\mu\text{M/ml}$: | + |
| " " | 0.2 - 1.0 $\mu\text{M/ml}$: | tr (trace) |
| " " | < 0.2 $\mu\text{M/ml}$: | - |

Lactic and succinic acids

| | | |
|---------------------|----------------------------|----|
| Concentration value | > 20 $\mu\text{M/ml}$: | ++ |
| " " | 10 - 20 $\mu\text{M/ml}$: | + |
| " " | 1 - 9 $\mu\text{M/ml}$: | tr |
| " " | < 1 $\mu\text{M/ml}$: | - |

Results

The results of morphological, biochemical, tolerance and antibiotic-disk-resistance tests with the 40 test strains are shown in Table 1. The proportion of strains that gave each result are shown in Table 2, and the results obtained with the 12 strains referred by the I.C.S.B. Taxonomic Subcommittee are shown in Table 3.

Preliminary Assignment of test strains to groups

The test strains submitted as B. melaninogenicus ss. melaninogenicus, B. oralis and B. ochraceus were divided into three groups for this analysis:

- (i) Strictly anaerobic strains that produced black or brown-pigmented colonies when grown on lysed-human-blood agar for up to one week were assigned to one group labelled B. melaninogenicus ss. melaninogenicus (23 strains).
- (ii) Strictly anaerobic, non-pigmented strains - B. oralis (11 strains). Three strains (ATCC 15930, see Holbrook and Duerden, 1974; VPI 7570A and 30) were referred to us as strains of B. oralis but produced black or brown-pigmented colonies and are included in our B. melaninogenicus group (above).
- (iii) Non-pigmented strains that were able to grow in 10% CO₂ in air - B. (Ristella) ochraceus (6 strains).

Morphology

Microscopic appearance. All strains were Gram-negative bacilli and many were pleomorphic. B. melaninogenicus ss. melaninogenicus and B. oralis strains were predominantly coccobacilli, occasionally arranged in short chains. B. ochraceus strains were long, slender bacilli with rounded or tapered ends and often with a central oval swelling.

Colony morphology. The colonies of B. melaninogenicus ss. melaninogenicus were 1-2 mm diameter, round, convex and opaque. After incubation for 48 h, they were typically light grey, becoming

brown after further incubation. The pigmentation varied between strains from light brown to almost black.

Colonies of B. oralis were 1-2 mm diameter, round, convex, opaque and grey; they tended to coalesce. After incubation for 7 days, the colonies of some strains (including VPI 5832) became light brown and were difficult to distinguish from the lighter-pigmented strains of B. melaninogenicus ss. melaninogenicus.

B. ochraceus strains typically produced two colony types: (a) 1 mm diameter, round or with an irregular edge, smooth, opaque and blue-grey; (b) 1 mm diameter, rhizoid, granular and ochre in colour.

Tolerance tests

(a) Bile salts. All strains of B. melaninogenicus ss. melaninogenicus and B. ochraceus were completely inhibited by the test concentrations of sodium taurocholate, sodium deoxycholate, and the combination of the two salts. Ten strains of B. oralis were also inhibited, but the growth of strain 7CM was not inhibited by either of the bile salts alone or by the combination.

(b) Dyes. All test strains were completely inhibited by the test concentrations of gentian violet and brilliant green, and only B. oralis strain 7CM was able to grow in the presence of ethyl violet. Twenty-one strains of B. melaninogenicus ss. melaninogenicus, 6 strains of B. oralis (including VPI 5832) and 2 of B. ochraceus (strains 1956C and VPI 2845) were completely

inhibited by Victoria blue 4R; however, 2 strains of B. melaninogenicus ss. melaninogenicus (WAL 2724 and one laboratory isolate), 5 strains of B. oralis (7CM, J1, NP333 and 2 laboratory isolates), and 4 strains of B. ochraceus (2467B, 10, 79B and 73) were able to grow in the presence of Victoria blue 4R.

Biochemical tests

None of the test strains produced indole, catalase or oxidase. All test strains produced acid from glucose, lactose, sucrose and maltose, but not from trehalose or mannitol.

Production of H₂S. Most strains produced H₂S, but some produced only small amounts and production by 2 strains of B. melaninogenicus ss. melaninogenicus and 2 strains of B. oralis (all laboratory isolates) was not detected.

Haemolysis. All strains of B. melaninogenicus ss. melaninogenicus produced some degree of haemolysis on human-blood agar. In most cases this was a zone of complete haemolysis around the colonies, but a number of strains, including WAL 2721, WAL 2724, GUI 1034 and VPI 7570A produced only a small zone of incomplete haemolysis. Two strains of B. oralis (J1 and 7CM) produced clear zones of complete haemolysis, 7 strains (including VPI 5832 and NP333) produced small zones of incomplete haemolysis and 2 laboratory isolates were non-haemolytic. None of the B. ochraceus strains were haemolytic.

Rhamnose fermentation. Six strains of B. oralis (including J1, 7CM, VPI 5832 and NP333), but no strains of

B. ochraceus and only one laboratory isolate of B. melaninogenicus ss. melaninogenicus produced acid from rhamnose.

Aesculin hydrolysis. Twelve of the 23 strains of B. melaninogenicus ss. melaninogenicus (strains ATCC 15930, GUI 1011, GUI 1034, VPI 7570A, 30 and 7 laboratory isolates), all of the test strains of B. oralis except 7CM, and all strains of B. ochraceus except 1956C hydrolysed aesculin.

Dextran hydrolysis. Fifteen strains of B. melaninogenicus ss. melaninogenicus (including VPI 4196, ATCC 15930, WAL 2724 and 30), all strains of B. ochraceus, and 5 strains of B. oralis (including NP333) hydrolysed dextran.

Gelatin digestion. Fifteen strains of B. melaninogenicus ss. melaninogenicus (including VPI 4196, VPI 7570A, ATCC 15930, GUI 1011 and GUI 1034), but only 3 laboratory isolates of B. oralis, digested gelatin disks. The other 8 strains of B. oralis and all 6 strains of B. ochraceus failed to digest the disks.

Lipase production. Strain 30 and seven other strains of B. melaninogenicus ss. melaninogenicus produced a lipase effect. This effect was not produced by any strain of B. oralis or B. ochraceus.

Antibiotic-Disk-Resistance Tests

All test strains were sensitive to erythromycin, rifampicin, lincomycin, clindamycin and chloramphenicol. They were all resistant to neomycin (10 µg), kanamycin (1000 µg and

30 µg) and bacitracin. Two strains, nos. 30 and 7CM, were resistant to neomycin (1000 µg), but all other strains were sensitive. All strains except ATCC 15930 were resistant to vancomycin. Five of the 6 strains of B. ochraceus were resistant to metronidazole; strain VPI 2845 was sensitive to metronidazole, but produced a smaller zone around the disk than the strictly anaerobic bacteroides organisms. All strains of B. oralis and B. ochraceus were sensitive to tetracycline, but 7 strains of B. melaninogenicus ss. melaninogenicus were resistant. Five strains of B. melaninogenicus ss. melaninogenicus (including WAL 2721) were resistant to penicillin. Only one strain of B. oralis (7CM) was resistant to penicillin, and all strains of B. ochraceus were sensitive. The five strains of B. melaninogenicus ss. melaninogenicus and one of B. oralis (strain 7CM) were also resistant to methicillin. Two strains of B. ochraceus (10 and 79B) were resistant to methicillin, but sensitive to penicillin. Fifteen strains of B. melaninogenicus ss. melaninogenicus (including VPI 4196, VPI 7570A, WAL 2724, GUI 1011 and GUI 1034) and 4 strains of B. oralis (including NP333) were sensitive to colistin, but all strains of B. ochraceus were resistant.

G.L.C. analysis

All the test strains produced acetic acid. All strains of B. melaninogenicus ss. melaninogenicus and all except two strains of B. oralis produced moderate amounts (++) but only two strains of B. ochraceus (1956C and 2467B) produced this amount; the remaining two strains of B. oralis (VPI 5832 and 7CM) and

4 strains of B. ochraceus produced minor amounts (+) of acetic acid.

Twelve strains of B. melaninogenicus ss. melaninogenicus, 3 strains of B. oralis and 2 strains of B. ochraceus produced minor amounts of propionic acid; a further 8 strains of B. melaninogenicus ss. melaninogenicus, 4 strains of B. oralis and one strain of B. ochraceus produced trace amounts.

No strains of B. oralis or B. ochraceus produced iso-butyric acid, and only 4 strains of B. melaninogenicus ss. melaninogenicus produced trace amounts of this acid. No strain produced n-butyric acid.

Two strains of B. melaninogenicus ss. melaninogenicus (WAL 2721 and a laboratory isolate) produced minor amounts of iso-valeric acid; a further 16 strains of B. melaninogenicus ss. melaninogenicus and 4 strains of B. oralis but only one strain of B. ochraceus produced trace amounts of this acid. No strain produced n-valeric acid.

One laboratory isolate of B. melaninogenicus ss. melaninogenicus produced a moderate amount of lactic acid; 4 strains produced minor amounts and 16 strains produced trace amounts of this acid. Two strains of B. oralis produced minor amounts and two produced trace amounts of lactic acid. Strains of B. ochraceus, however, did not produce lactic acid except for a trace amount produced by strain 1956C.

All strains produced succinic acid; 14 strains of B. melaninogenicus ss. melaninogenicus, 6 strains of B. oralis

and 3 strains of B. ochraceus produced moderate amounts and the remainder produced minor amounts of this acid.

The patterns of results indicate that differences between "-" and "trace" or "trace" and "+" results are probably less significant than differences between "-", "+" and "++" results.

Comments

As a result of these studies with 40 test strains of B. melaninogenicus ss. melaninogenicus, B. oralis and B. ochraceus, the three groups were distinguished as follows:

B. melaninogenicus ss. melaninogenicus strains were strict anaerobes that produced brown, or occasionally black, colonies on media containing blood. They produced acid from glucose (and lactose, sucrose and maltose). They did not produce indole. In general, in disk-tests, they were sensitive to neomycin (1000 µg disk), rifampicin and metronidazole, and they were resistant to kanamycin (1000 µg disk). They were inhibited in tolerance tests with two bile salts (separately and in combination), and they were inhibited by each of the four test dyes. Most strains were sensitive to penicillin. The results of tests for hydrolysis of aesculin and dextran, gelatin liquefaction, lipase production, and resistance to colistin varied between strains; there was no apparent relationship between the results of these tests.

In G.L.C. analysis, the test strains of this subspecies produced moderate amounts of acetic and succinic acids from glucose; propionic, iso-valeric and lactic acids were variable minor products.

B. oralis strains were strict anaerobes that failed to produce pigment, although the colonies of some strains on lysed-blood agar became pale brown after incubation for 7 days. They were essentially similar to B. melaninogenicus ss. melaninogenicus strains and four laboratory isolates were indistinguishable from B. melaninogenicus ss. melaninogenicus except that they did not produce pigment. The four referred strains of B. oralis and three laboratory isolates were distinguished by the ability to produce acid from rhamnose and five of these strains were able to grow in the presence of Victoria blue 4R. The results of G.L.C. analysis were generally indistinguishable from the results obtained with B. melaninogenicus ss. melaninogenicus strains.

B. ochraceus strains were clearly distinguished by their ability to grow in air plus CO₂. They produced acid from glucose (and lactose, sucrose and maltose) but not from rhamnose, and did not produce indole. They were generally sensitive to disks of neomycin (1000 µg) and rifampicin, but resistant to metronidazole, kanamycin (1000 µg), and colistin. They hydrolysed aesculin (except one strain) and dextran. In G.L.C. analysis, they produced smaller amounts of acetic acid than the B. melaninogenicus ss. melaninogenicus and B. oralis groups;

propionic acid was a variable minor product; they did not produce lactic, iso-butyric or iso-valeric acids.

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TABLE 2

Percentage of specified results obtained
with the three groups of test strains

| Test | Result | % of test strains with the given result | | | | |
|---|--------|---|------------------|-------------------------|--|------------------------|
| | | B.mel. ss.mel. (23) | B.oralis (11) | B.och- raceus (6) | all non- pigmented strains (17) | all strains (40) |
| Growth in O ₂ + CO ₂ | + | 0 | 0 | 100 | 35.3 | 15 |
| Pigment production | + | 100 | 0 | 0 | 0 | 57.5 |
| <u>Tolerance tests:</u> | | | | | | |
| Taurocholate (T) | I | 100 | 90.9 | 83.3 | 94.1 | 95 |
| Deoxycholate (D) | I | 100 | 100 | 100 | 100 | 100 |
| T + D | I | 100 | 100 | 100 | 100 | 100 |
| Victoria Blue 4R | I | 91.3 | 54.5 | 33.3 | 47.1 | 72.5 |
| ethyl violet | I | 100 | 90.9 | 100 | 94.1 | 97.5 |
| gentian violet | I | 100 | 100 | 100 | 100 | 100 |
| brilliant green | I | 100 | 100 | 100 | 100 | 100 |
| <u>Antibiotic-disk- resistance tests:</u> | | | | | | |
| neomycin (1000 µg) | S | 95.7 | 90.9 | 100 | 94.1 | 95 |
| kanamycin (1000 µg) | R | 100 | 100 | 100 | 100 | 100 |
| rifampicin | S | 100 | 100 | 100 | 100 | 100 |
| penicillin | S | 78.3 | 90.9 | 100 | 94.1 | 85 |
| methicillin | S | 78.3 | 90.9 | 66.6 | 82.4 | 80 |
| metronidazole | S | 91.3 | 90.9 | 100 | 94.1 | 92.5 |
| tetracycline | S | 69.6 | 100 | 100 | 100 | 82.5 |
| colistin | S | 65.2 | 36.4 | 0 | 23.5 | 47.5 |
| bacitracin | R | 100 | 100 | 100 | 100 | 100 |
| vancomycin | R | 95.7 | 100 | 100 | 100 | 97.5 |
| <u>Biochemical tests:</u> | | | | | | |
| H ₂ S production | + | 91.3 | 81.8 | 100 | 88.2 | 90 |
| Haemolysis | + | 100 | 81.8 | 0 | 52.9 | 80 |
| Indole production | + | 0 | 0 | 0 | 0 | 0 |

TABLE 2 (continued)

| Test | Result | % of test strains with the given result | | | | |
|-------------------------|--------|---|------------------|-------------------------|--|------------------------|
| | | B.mel ss.mel (23) | B.oralis (11) | B.och- raceus (6) | all non- pigmented strains (17) | all strains (40) |
| Aesculin hydrolysis | + | 52.2 | 90.9 | 83.3 | 88.2 | 67.5 |
| Dextran hydrolysis | + | 65.2 | 45.5 | 100 | 64.7 | 65 |
| Gelatin digestion | + | 65.2 | 27.3 | 0 | 17.7 | 45 |
| Lipase production | + | 34.8 | 0 | 0 | 0 | 20 |
| <u>Fermentation of:</u> | | | | | | |
| glucose | + | 100 | 100 | 100 | 100 | 100 |
| lactose | + | 100 | 100 | 100 | 100 | 100 |
| maltose | + | 100 | 100 | 100 | 100 | 100 |
| sucrose | + | 100 | 100 | 100 | 100 | 100 |
| rhamnose | + | 4.3 | 63.6 | 0 | 41.2 | 20 |
| trehalose | + | 0 | 0 | 0 | 0 | 0 |
| mannitol | + | 0 | 0 | 0 | 0 | 0 |
| <u>G.L.C. analysis:</u> | | | | | | |
| acetic | + | 0 | 18.2 | 66.7 | 35.3 | 15 |
| " | ++ | 100 | 81.8 | 33.3 | 64.7 | 85 |
| propionic | - | 13 | 36.4 | 50 | 41.2 | 25 |
| " | tr | 34.8 | 36.4 | 16.7 | 29.4 | 32.5 |
| " | + | 52.2 | 27.2 | 33.3 | 29.4 | 42.5 |
| iso-butyric | - | 82.6 | 100 | 100 | 100 | 90 |
| " | tr | 17.4 | 0 | 0 | 0 | 10 |
| n-butyric | - | 100 | 100 | 100 | 100 | 100 |
| iso-valeric | - | 21.7 | 63.6 | 83.3 | 70.6 | 42.5 |
| " | tr | 69.5 | 36.4 | 16.7 | 29.4 | 52.5 |
| " | + | 8.7 | 0 | 0 | 0 | 5 |
| n-valeric | - | 100 | 100 | 100 | 100 | 100 |
| lactic | - | 8.7 | 63.6 | 83.3 | 70.6 | 35 |
| " | tr | 69.6 | 18.2 | 16.7 | 17.6 | 47.5 |
| " | + | 17.4 | 18.2 | 0 | 11.8 | 15 |
| " | ++ | 4.3 | 0 | 0 | 0 | 2.5 |
| succinic | + | 39.1 | 45.5 | 50 | 47.1 | 42.5 |
| " | ++ | 60.9 | 54.5 | 50 | 52.9 | 57.5 |

APPENDIX V

Publications from this thesis

A COMPARISON OF SOME CHARACTERISTICS OF REFERENCE STRAINS OF *BACTEROIDES ORALIS* WITH *BACTEROIDES MELANINOGENICUS*

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Summary—*Bacteroides oralis* strain ATCC no. 15930 was compared with three reference strains of *B. melaninogenicus* (NCTC nos. 9336, 9337 and 9338) in terms of their microscopic and colonial morphology, their behaviour in a series of simple biochemical tests and disc antibiotic sensitivity tests. The *B. oralis* strain produced black pigmented colonies on blood agar and the extracted pigment appeared identical with that produced by the *B. melaninogenicus* strains. The results of the other tests were also essentially similar. Unless valid reasons can be given for the separation of these reference strains into two species, it is suggested that *B. oralis* strain ATCC no. 15930 be re-classified as *B. melaninogenicus*.

Bacteroides oralis and *B. melaninogenicus* are two species commonly isolated in large numbers from the human mouth. *B. melaninogenicus* was first described by Oliver and Wherry (1921) as a Gram-negative anaerobic bacillus that produces black pigmented colonies on blood agar. This characteristic appearance has been regarded as highly specific and remains the sole basis for the differentiation of *B. melaninogenicus* from other *Bacteroides* species. The species *B. oralis* was proposed by Loesche, Soeransky and Gibbons (1964) and is included in three identification schemes for Gram-negative non-sporing anaerobes (Loesche and Gibbons, 1965; Sutter and Finegold, 1971; Holdeman and Moore, 1973). An extensive list of its characteristics is published in the Anaerobe Laboratory Manual (Holdeman and Moore, 1973), and these are similar to *B. melaninogenicus* ss. *melaninogenicus* except that *B. oralis* does not produce pigment.

As part of a larger study of the identification and classification of Gram-negative, anaerobic, non-sporing bacteria, the only currently available reference strain of *B. oralis* (ATCC no. 15930) and three reference strains of *B. melaninogenicus* (NCTC nos. 9336, 9337, 9338) were obtained from the American Type Culture Collection (12301 Parklawn Dr., Rockville, Md. 20852) and the National Collection of Type Cultures (Central Public Health Laboratory, Colindale Avenue, London, NW9 5HT), respectively. These strains were maintained by serial subculture at 10–12 day intervals in pre-steamed Robertson's cooked meat broth (Cruickshank, 1968). Cultures were incubated for 48 hr at 37°C in an atmosphere of 90 per cent H₂ and 10 per cent CO₂ in a Baird & Tatlock Ltd. (BTL) anaerobic jar equipped with three room-temperature catalyst sachets following the method of Collee *et al.*

(1972). These stock cultures were then held at room temperature on the bench without further anaerobic measures. Cellular morphology was regularly checked by examination of Gram-stained smears and the purity of cultures checked by aerobic and anaerobic subculture on blood agar. All tests were performed by the methods of Cruickshank (1968) unless otherwise stated.

Growth characteristics, colonial morphology, and the appearance of haemolysis and pigmentation were observed on nutrient agar media (Oxoid Columbia Agar) containing: (i) 5 per cent human blood, (ii) 5 per cent lysed human blood, (iii) 5 per cent horse blood (Wellcome Laboratories) or (iv) 5 per cent lysed horse blood. Lipase and phospholipase activity were detected by growth on egg yolk agar and colonies from this medium were tested for catalase and oxidase production. Indole production in cooked meat broth was tested using Ehrlich's reagent after benzol extraction and H₂S production was detected by lead acetate paper strips. Charcoal-gelatin discs (Oxoid) were added to cooked meat broth cultures to detect proteolysis and these cultures were only discarded as negative after incubation for 2 weeks. Aesculin hydrolysis was determined in cooked meat broth with aesculin added to give a final concentration of 1 per cent. After incubation for 1 week, hydrolysis was indicated by a dense, black colour after adding 1 ml of a 1 per cent solution of ferric ammonium citrate.

Carbohydrate fermentation was determined in thioglycollate medium (BBL) without dextrose or indicator and with added yeast extract (0.2 per cent), haemin (5 µg ml⁻¹), menadione (0.25 µg ml⁻¹), and glucose, lactose, sucrose or maltose added to give a final concentration of 1 per cent. After incubation for 1

week, the pH of the cultures was measured with a Pye Unicam Model 292 pH meter (Pye, Cambridge, England) and a Pye Ingold combined glass and reference electrode No. 401-S/160. Fermentation was defined as a fall of >0.5 pH unit below the pH value of the inoculated carbohydrate-free control (Rutter, 1970).

The antibiotic disc sensitivity test was modified from Sutter and Finegold (1971). One drop (0.02 ml) of a 48-hr cooked meat broth culture was spread over the surface of a 5 per cent human blood agar plate. Four discs were placed on each plate and the diameters of inhibition zones were measured as soon as good growth was visible, usually after 48 hr. The discs contained neomycin 1000 μg and 10 μg , kanamycin 1000 μg , erythromycin 60 μg , penicillin 1.5 units, tetracycline 10 μg , colistin 10 μg , rifampicin 10 μg , lincomycin 2 μg , bacitracin 0.1 unit, vancomycin 15 μg and chloramphenicol 10 μg .

The black pigment was extracted from cells grown on 5 per cent human blood agar for 10 days. The cells were washed thrice in distilled water and disrupted by ultrasonication. After ultracentrifugation, the pigment in aqueous solution was examined by spectrophotometry (Duerden, 1974).

B. oralis and *B. melaninogenicus* strains were all non-motile, non-sporing, Gram-negative, cocco-bacilli with some short chains. Occasionally a longer form of *B. oralis* with pointed ends was seen. Both forms were seen in preparations made from single colonies on human blood agar. After incubation for 48 hr on human blood agar, the colonies of each strain were small (<1 mm, dia.), circular, convex and translucent, with an entire edge. The *B. oralis* strain grew more slowly than the *B. melaninogenicus* strains. Zones of complete haemolysis were seen around colonies of *B. melaninogenicus* strains after 2–5 days and around colonies of *B. oralis* after 5–7 days. The colonies initially became grey; 24 hr after haemolysis appeared, the colonies became black. Pigmentation developed 24–48 hr earlier on lysed human blood agar but more slowly on media containing horse blood. All of the strains produced pigment within 7 days on media containing either human or horse blood. Spectrophotometry of aqueous solutions of pigment extracted by ultrasonication from black colonies of *B. oralis* and *B. melaninogenicus* strains showed a similar pattern for each strain. There was a broad peak in the 370–450 nm band with a maximum at 410 nm; this was not so with preparations derived from the medium control.

The cultural and biochemical characteristics of the strains are shown in Table 1. All of the strains produced H_2S and digested gelatin and none of them produced oxidase or catalase. *B. oralis* strain 15930 and *B. melaninogenicus* strain 9338 produced a lipase and a phospholipase. Indole was produced by all of the *B. melaninogenicus* strains but not by *B. oralis* strain 15930. Aesculin was hydrolysed by *B. oralis* strain 15930 but not by any of the *B. melaninogenicus* strains. *B. oralis* strain 15930 fermented glucose, lactose, maltose, and sucrose, whereas *B. melaninogenicus* strains

9336 and 9338 fermented glucose, sucrose and maltose, but not lactose. *B. melaninogenicus* strain 9337 was non-saccharolytic.

The results of antibiotic disc sensitivity tests obtained with the four strains were essentially similar (Table 2). The only differences between *B. oralis* strain 15930 and the three strains of *B. melaninogenicus* were that strain 15930 was resistant to bacitracin and sensitive to vancomycin. *B. melaninogenicus* strain 9337 and *B. oralis* strain 15930 were resistant to colistin, whereas *B. melaninogenicus* strains 9336 and 9338 were sensitive.

In the currently accepted definition (Loesche and Gibbons, 1965; Holdeman and Moore, 1973), the species *Bacteroides melaninogenicus* is a Gram-negative, non-motile, non-sporing, anaerobic bacillus or coccobacillus that produces black pigmented colonies when grown on blood agar. All strains that produce pigment in this way are classified as *B. melaninogenicus*. This has been challenged by Tracy (1969) who showed that *Bacteroides*-like organisms generally can produce a black colloidal precipitate in certain fluid media. Duerden (1974) has recently attributed this to the production of H_2S under cultural conditions that provide ferrous ions leading to a precipitate of ferrous sulphide. The true pigment produced in colonies grown on blood agar is a different and a specific product of *B. melaninogenicus* strains. The species comprises a heterogeneous group of strains with wide variations in biochemical properties between strains and some workers have identified sub-species on the basis of these properties (Holdeman and Moore, 1973). Our observation that the reference strain of *B. oralis* ATCC 15930, produced black pigment when grown on human or horse blood agar indicates that this strain should be classified as *B. melaninogenicus* according to the above definition. Sharpe (1971) reported a personal communication from Dr. M. P. Bryant that a strain derived from *B. oralis* ATCC strain 15930 produced black colonies; Sharpe was unable to reproduce that finding. However, recent observations of pigment production by *B. oralis* ATCC strain 15930 on lysed blood agar have prompted other workers to consider re-classification of the strain (E. P. Cato, 1974, personal communication). Moreover, other major characteristics of all of the sub-species of *B. melaninogenicus* are sensitivity to penicillin and to high concentrations of neomycin (Sutter and Finegold, 1971), and the ability to digest gelatin; these characteristics are shared by strain 15930. Considerable differences remain between the four strains in terms of their carbohydrate fermentation reactions and lipase production, but these differences between strain 15930 and the reference strains of *B. melaninogenicus* are no greater than the differences observed between strains accepted as *B. melaninogenicus*.

Holdeman and Moore (1973) differentiate between three sub-species of *B. melaninogenicus* (ss. *melaninogenicus*, ss. *intermedius* and ss. *asaccharolyticus*). The reference strains 9336 and 9338 accordingly correspond with *B. melaninogenicus* ss. *intermedius*, and

Table 1. Results of cultural and biochemical tests with strains of *B. oralis* and *B. melaninogenicus*

| Test or observation | Results obtained with strain* | | | |
|------------------------------------|--------------------------------|--|--|--|
| | <i>B. oralis</i> ATCC 15930 | <i>B. melaninogenicus</i> NCTC 9336 | <i>B. melaninogenicus</i> NCTC 9337 | <i>B. melaninogenicus</i> NCTC 9338 |
| Black pigment on blood agar | + | + | + | + |
| Haemolysis on blood agar at 48 hr | — | — | — | + |
| Haemolysis on blood agar at 7 days | + | + | + | + |
| Phospholipase production | + | — | — | + |
| Lipase production | + | — | — | + |
| Oxidase production | — | — | — | — |
| Catalase production | — | — | — | — |
| H ₂ S production | + | + | + | + |
| Digestion of Gelatin | + | + | + | + |
| Indole production | — | — | — | — |
| Aesculin hydrolysis | + | + | + | + |
| Glucose fermentation | + | + | — | + |
| Lactose fermentation | + | — | — | — |
| Maltose fermentation | + | + | — | + |
| Sucrose fermentation | + | + | — | + |
| Motility | — | — | — | — |
| Aerobic growth | — | — | — | — |

* + = positive result; — = negative result.

Table 2. Results of antibiotic disc sensitivity tests with strains of *B. oralis* and *B. melaninogenicus*

| Antibiotic used* | Diameter of inhibition zone obtained with strain† | | | |
|-------------------------|---|--|--|--|
| | <i>B. oralis</i> ATCC 15930 | <i>B. melaninogenicus</i> NCTC 9336 | <i>B. melaninogenicus</i> NCTC 9337 | <i>B. melaninogenicus</i> NCTC 9338 |
| Neomycin (1000 µg) | ++ | ++ | ++ | ++ |
| Neomycin (10 µg) | R | R | R | R |
| Kanamycin (1000 µg) | R | R | R | R |
| Penicillin (1.5 units) | S | S | S | S |
| Erythromycin (60 µg) | S | S | S | S |
| Tetracycline (10 µg) | S | S | S | S |
| Colistin (10 µg) | R | ++ | R | ++ |
| Rifampicin (10 µg) | S | S | S | S |
| Lincomycin (2 µg) | S | S | S | S |
| Bacitracin (0.1 units) | R | + | + | ++ |
| Vancomycin (15 µg) | ++ | R | R | ++ |
| Chloramphenicol (10 µg) | S | S | ++ | S |

* The amount of antibiotic contained in a disc is given in brackets.

† R = diameter of inhibition zone < 15 mm; + = diameter of inhibition zone 15 × 25 mm; ++ = diameter of inhibition zone 25 × 35 mm; S = diameter of inhibition zone > 35 mm.

strain 9337 with *B. melaninogenicus* ss. *asaccharolyticus*; strain 15930 corresponds with *B. melaninogenicus* ss. *melaninogenicus*. The characteristics of *B. melaninogenicus* ss. *melaninogenicus* are essentially similar to the reported characteristics of *B. oralis* (Loesche and Gibbons, 1965; Holdeman and Moore, 1973) except for the property of pigment production that appears to be the sole basis for differentiation between the two species. As we have shown that the *B. oralis* strain can produce pigment, we suggest that the reference strain "*B. oralis* ATCC no. 15930" should be re-classified as a strain of *B. melaninogenicus*.

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The Characterization of Clinically Important Gram Negative Anaerobic Bacilli by Conventional Bacteriological Tests

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One hundred and sixty-five reference strains and laboratory isolates of Gram negative, non-sporing, anaerobic bacilli were subjected to a series of simple laboratory tests that were initially selected for their discriminatory value. Conventional biochemical tests, tests of resistance to antibiotics, and tolerance to dyes and bile salts were included. These tests allowed a clear separation of strains into three main groups: *Bacteroides fragilis*, *B. melaninogenicus* and *Fusobacterium* spp. Certain tests were found useful for identifying recognized subspecies of *B. fragilis* and *B. melaninogenicus*. A scheme for the identification of unknown laboratory isolates of Gram negative anaerobic bacilli is presented.

BACTERIA OF THE *Bacteroides-Fusobacterium* group are significant anaerobic pathogens in many clinical conditions, notably periodontal disease (MacDonald, Gibbons & Socransky, 1960; Socransky, 1970), bronchiectasis and lung abscess (Bartlett & Finegold, 1972), wound infections following abdominal and pelvic surgery (Finegold, 1974), chronic pelvic infections in women (Thadepalli, Gorbach & Keith, 1973) and post-operative bacteraemia (Chow & Guze, 1974). There are, however, technical problems relating to their culture and identification. The present classification of the bacteroides-like bacteria is confused and most routine diagnostic laboratories are unable to identify the organisms beyond the level of '*Bacteroides* spp'.

Recent advances in techniques of handling and anaerobic culture of these bacteria—e.g. the adoption of a standard anaerobic procedure (Collee, *et al.*, 1972), the addition of 10% carbon dioxide to the anaerobic atmosphere (Watt, 1973), the use of lysed blood agar (Finegold, Sugihara & Sutter, 1971), and the addition of haemin and vitamin K as growth factors for *Bacteroides melaninogenicus* (Gibbons & MacDonald, 1960)—have overcome many of the difficulties previously encountered in isolating them in pure culture. It is now possible to recover them quantitatively on solid media (Watt, 1972) and to obtain good growth in liquid media by conventional bacteriological techniques at the bench.

Our objective in the present study was to produce a scheme for the identification of isolates of the bacteroides group from which a simple approach might be derived for

use in the routine diagnostic laboratory. We have used conventional bacteriological methods to characterize a series of 165 bacteroides-like organisms including standard reference strains, referred strains and new isolates from clinical specimens and from healthy subjects.

Materials and Methods

Cultures were regularly checked for cell morphology and purity by examination of Gram-stained smears and by aerobic and anaerobic subculture on human-blood agar.

Culture inocula

One drop (0.02 ml) of a 48-h cooked meat broth culture was used to seed each tube and either one drop or one loopful (c. 0.01 ml) to seed each plate of medium.

Anaerobic incubation

The standard anaerobic procedure of Collee *et al.* (1972) was followed with anaerobic jars supplied by Baird and Tatlock Ltd (BTL), each fitted with three catalyst sachets. All incubation was at 37° in an atmosphere of 90% H₂ and 10% CO₂.

Incubation under low oxygen tension

An anaerobic jar without catalyst was evacuated until the internal atmospheric pressure was reduced to 180 mmHg. The pressure was then equilibrated with CO₂, i.e. c. 5% O₂ and 80% CO₂. The jars were incubated at 37° for 48 h.

Organisms

The following strains were obtained from the National Collection of Type Cultures (NCTC), Central Public Health Laboratory, Colindale Avenue, London, NW9 5HT: *Bacteroides fragilis* (*B. fragilis* ss. *fragilis*) 9343, 9344, 10581, 10584 and 8560; *B. thetaiotaomicron* (*B. fragilis* ss. *thetaitaomicron*) 10582; *B. vulgatus* (*B. fragilis* ss. *vulgatus*) 10583; *B. melaninogenicus* 9336, 9337 and 9338; *Fusobacterium polymorphum* 10562; *F. necrogenes* 10723; *F. (Bacteroides; Sphaerophorus) necrophorum* 10575, 10576 and 10577; *Leptotrichia buccalis* 10249; *L. dentium* 10206; *B. necrophorus* 7155 (see Barnes & Goldberg, 1968; Lapage, 1972). *B. oralis* 15930 (*B. melaninogenicus*, see Holbrook & Duerden, 1974) was from the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, Md. 20852, U.S.A. *B. fragilis* ss. *vulgatus* ATCC 8482, *B. fragilis* ss. *ovatus* ATCC 8483, *B. fragilis* ss. *thetaitaomicron* ATCC 8492, and *B. fragilis* ss. *distasonis* ATCC 8503 were from Dr Ella M. Barnes, Agricultural Research Council, Food Research Institute, Colney Lane, Norwich, NOR 70F. *B. ochraceus* VPI 2845 and *B. melaninogenicus* ss. *melaninogenicus* VPI 4196 were from Dr Elizabeth Cato, Virginia Polytechnic Institute and State University, Blacksburg, Va. 24060, U.S.A. *B. oralis* NP 333 was from Mr G. H. Bowden, London Hospital Medical College, Dental School, Turner St., London, E1 2AD. Three strains of *B. melaninogenicus* (strains 2296, 3502 and 3586) were from Dr R. Wiseman, Bangour General Hospital, W. Lothian. Four strains of *B. corrodens*

(strains 124, NC-1, NC-2, and NCL 20) were from Dr A. L. James, Newcastle Polytechnic, Newcastle upon Tyne, England. Thirty-nine strains were isolated from clinical specimens at the Royal Infirmary, Edinburgh; 50 strains were from clinical specimens at the Western General Hospital, Edinburgh; 26 strains were isolated from normal human faeces in this laboratory; and 17 strains were isolated from human gingival crevice material and dental plaque sampled at the Dental Hospital, Edinburgh. A total of 165 strains was examined.

Media

These were either freshly prepared or steamed for 30 min and promptly cooled just before use. Media containing menadione could not be steamed and were always freshly prepared.

The basic liquid media were Robertson's cooked meat broth (Cruickshank, 1968, modified by Watt, 1973) and thioglycollate medium without dextrose or indicator (Baltimore Biological Laboratories); various combinations of chemicals were added (see below). All solid media were prepared with Columbia agar base (Oxoid). Blood agar contained 5% outdated human blood provided by the Regional Blood Transfusion Service, Edinburgh; each 500 ml volume of the human blood preparation contained 2 g disodium citrate and 1.7 g dextrose in 70 ml of water added to 430 ml of whole blood; lysed blood agar was prepared by treating the blood with saponin before incorporating it in the medium (Cruickshank, 1968).

Growth factors. The following chemicals were added as indicated for the growth of the more fastidious organisms. Stock solution of haemin (haematin hydrochloride, BDH) contained 500 µg/ml in 0.01 N-NaOH and stock solution of menadione (Sigma, London) contained 100 µg/ml in distilled water (see Barnes & Impey, 1971); these filter-sterilized solutions were added aseptically to (cooled) autoclaved media to give final concentrations of haemin 5 µg/ml and menadione 1 µg/ml. A filter-sterilized solution of glucose in distilled water was added to (cooled) autoclaved media to give a final concentration of 1%. When required 0.25% yeast extract (Oxoid) and 0.25% sodium succinate (BDH) were added to media before autoclaving.

Characterization of strains

Morphology. In general, observations were recorded after growth for 48 h on blood agar; colonies of strains that grew slowly were described as soon as they were visible. Cell morphology was noted in Gram-stained smears from cultures grown for 48 h (i) on blood agar and (ii) in cooked meat broth.

Haemolysis on blood agar was observed after incubation for 48 h and 1 week.

Pigment production. Strains were observed for production of black-pigmented colonies on lysed blood agar with menadione after incubation for up to 2 weeks.

Motility. A wet film prepared from a 48 h cooked meat broth culture was examined by phase-contrast microscopy. Strains were also stab inoculated into semi-solid agar

(motility test medium) which was then incubated anaerobically until growth was visible. The medium contained 0.2% agar (Oxoid no. 2) in thioglycollate medium with yeast extract, haemin and menadione.

Lipase activity. This was detected by observing the effect of growth on egg yolk agar (EYA) (Cruickshank, 1968), supplemented with sodium succinate, yeast extract, haemin and menadione.

Oxidase test. A freshly prepared 1% solution of tetramethyl-*p*-phenylene-diamine dihydrochloride in distilled water was poured on to a 1-week culture on supplemented egg yolk agar. A blue colour indicated oxidase production.

Catalase test. A 1-week culture on supplemented egg yolk agar was flooded with 10% hydrogen peroxide solution; a stream of bubbles arose from colonies of catalase-producing organisms.

Hydrogen sulphide production. A strip of lead acetate paper was suspended during incubation in the neck of a tube of a cooked meat broth culture. Blackening of the paper indicated H₂S production.

Nitrate reduction. This was tested in thioglycollate medium with yeast extract, haemin, menadione and KNO₃ (200 µg/ml). The presence of nitrite ions was indicated by a deep red colour when 0.5 ml of Nitrate Solution A and 0.5 ml of Nitrate Solution B (Cruickshank, 1968) were added to 48-h cultures.

Indole production. Indole was detected by adding 0.5 ml of benzol to the liquid supernate of a 48-h cooked meat broth culture and then adding a few drops of Ehrlich's Reagent. A pink colour indicated the presence of indole. Positive and negative control strains were included in each batch of tests.

Gelatinase test. A charcoal-gelatin disc in a cooked meat broth culture was observed for digestion of the disc during incubation for 2 weeks. In view of difficulties encountered with commercially available discs, the discs were prepared in our own laboratory by a modification of Kohn's method (Kohn, 1953): 12.5 g gelatin (Difco) was dissolved in 100 ml of nutrient broth (Oxoid no. 2); 5 g of finely powdered charcoal was added and the mixture was poured into metal Petri dishes and allowed to solidify at 4°. The charcoal-gelatin was held in 10% formalin at room temperature for 5 days and then cut into discs 1 cm in diameter. The discs were washed in running tap water for 48 h at 4° and pasteurized by heating at 70° in sterile distilled water for 20 min.

Aesculin hydrolysis. The organisms were grown for 48 h in cooked meat broth containing 1% aesculin. If aesculin was hydrolysed a black discoloration developed when 0.5 ml of a 1% aqueous solution of ferric ammonium citrate was added.

Dextran hydrolysis. Strains were grown for one week on a medium containing 0.5% dextran T40 (Pharmacia, Uppsala, Sweden), 0.5% blue dextran 2000 (Pharmacia), 0.2% glucose, 0.25% sodium succinate, 0.25% yeast extract, 5 µg/ml haemin and

1 µg/ml menadione in Columbia agar base (Oxoid) (modified from Staat, Gawronski & Schachtele, 1973). Hydrolysis was indicated by the development of a zone of clearing around colonies growing on the blue medium.

Carbohydrate-fermentation tests. Filter-sterilized 20% solutions of glucose, lactose, sucrose, maltose, rhamnose, trehalose and mannitol in distilled water were added separately to tubes of pre-steamed cooked meat broth, or thioglycollate medium with haemin, menadione and yeast extract, to give a final concentration of 1% of the test sugar. The tubes were seeded with 1 drop (0.02 ml) from a 48-h cooked meat broth culture and incubated anaerobically for 48 h. Slow-growing strains were also incubated for 1 week. The final pH was measured with a Pye Unicam model 292 pH meter and a Pye Ingold combined Glass and Reference Electrode no. 401-S/160 after aerobic exposure on the bench for at least 1 h. Controls included cultures of the test strains in plain cooked meat broth or thioglycollate medium and tubes of uninoculated (but incubated) 1% sugar media. The test was regarded as positive if the pH fell >0.5 unit below that of the uninoculated control and >0.5 pH unit in comparison with the value for the 48-h culture in plain cooked meat broth (see Rutter, 1970).

Tolerance tests

(a) *Inhibition of growth by bile salts.* Each strain was seeded on a series of four nutrient agar test media supplemented with sodium succinate, haemin and menadione, and containing (i) no bile salt (control); (ii) 0.5% sodium taurocholate (BDH); (iii) 0.1% sodium deoxycholate (BDH); or (iv) 0.5% sodium taurocholate plus 0.1% sodium deoxycholate. The plates were examined for growth after 48 h, or longer for fastidious strains.

(b) *Sensitivity to dyes.* Each strain was seeded on a series of five nutrient agar media supplemented with sodium succinate, yeast extract, haemin and menadione, and containing (i) no dye (control); (ii) brilliant green (1 in 80 000); (iii) Victoria blue 4R (1 in 80 000); (iv) gentian violet (1 in 100 000); or (v) ethyl violet (1 in 80 000). The plates were examined for growth after 48 h. Each dye was initially prepared in aqueous solution and the concentrations above are those finally achieved in the test medium.

(c) *Antibiotic resistance tests.* The test strains were grown on fresh blood agar and tested for resistance to antibiotics by the disc-diffusion method (modified from Sutter & Finegold, 1971). The plates were seeded by spreading 0.02 ml of a 48-h cooked meat broth culture on the surface with a glass spreader. The diameters of zones of inhibition were measured after either 24 or 48 h, as soon as good growth was visible.

Discs containing neomycin sulphate 1000 µg, kanamycin sulphate 1000 µg, benzyl penicillin 1.5 units, erythromycin ethyl succinate 60 µg, colistin sulphate 10 µg, rifampicin (Rimactane) 15 µg, vancomycin 15 µg, and chloramphenicol 10 µg were prepared in our laboratory. Discs containing methicillin 10 µg, lincomycin 2 µg, clindamycin 2 µg, bacitracin 0.1 unit, neomycin 10 µg and kanamycin 30 µg were obtained from Mast Laboratories. *B. fragilis* NCTC 9343 was selected as the reference strain for tests. It was tested in parallel experiments with a standard Oxford staphylococcus that was sensitive to neomycin, kanamycin, penicillin, methicillin,

erythromycin, rifampicin, lincomycin, clindamycin, vancomycin and chloramphenicol but resistant to colistin and bacitracin. The diameters of zones of inhibition were grouped in four grades: <15 mm; 16–25 mm; 26–35 mm; >35 mm. The grades obtained with *B. fragilis* NCTC 9343 were classified as resistant (R) or sensitive (S) by comparison with the grades obtained with the Oxford staphylococcus. The grades obtained with test strains were compared with those from *B. fragilis* NCTC 9343 and classified as 'resistant' or 'sensitive' in relation to that reference strain.

Results

Cell morphology

All strains were Gram negative, non-sporing, non-motile, obligately anaerobic bacteria, except *L. dentium* NCTC 10206 which was a Gram-variable facultative aerobe with many Gram positive cells in young cultures. Pleomorphism was common. Cell shape varied from filamentous to cocco-bacillary, often in the same smear; some strains formed chains; some had pointed ends and others rounded ends. Cell morphology varied further with the culture medium and had little discriminatory value. However, many *B. melaninogenicus* strains were predominantly cocco-bacillary and many *Fusobacterium* spp. showed long filamentous forms.

Colony morphology

Colonies on blood agar differed in size from pin-point to 3–4 mm in diameter. Some but not all *Fusobacterium* spp. produced rhizoid colonies which were never produced by *Bacteroides* spp. Black-pigmented colonies with haloes of haemolysis were typical of *B. melaninogenicus*. Many strains gave a small zone of incomplete haemolysis after prolonged incubation, but a wider zone of complete haemolysis was characteristic of *B. melaninogenicus* and some *Fusobacterium* spp. (Tables 1a, b, c).

The test strains were divided into three groups on the basis of the results of biochemical, tolerance and antibiotic resistance tests. Strains that broadly resembled *B. fragilis* NCTC 9343 were designated 'B-strains'; strains that produced black pigmented colonies on blood agar were regarded as *B. melaninogenicus* strains (Duerden, 1975) and designated 'M-strains'; the remaining strains that included reference strains of *Fusobacterium* spp. and *Leptotrichia* spp. and a small heterogeneous group of laboratory isolates that did not conform with the broad characteristics of the other two groups were designated 'F-strains'.

Results of biochemical tests

None of the test strains produced oxidase and only *L. dentium* NCTC 10206 produced catalase.

The 'B-strains'. The 20 patterns of results in a series of biochemical tests on 107 B-strains are listed in Table 1a and are coded Bi-Bxx. Almost all results were clearly positive or negative. Haemolysis, however, was variable and at most weak and incomplete with B-strains, and a few variable results were obtained in tests for sucrose fermentation.

TABLE 1A
Results of biochemical tests with non-pigmented Bacteroides organisms (B-strains)

| Test | Pattern of results* | | | | | | | | | | | | | | | | | | | |
|---------------------------------------|---------------------|------------|-----------|-----------|------------|------------|-----------|-----------|-----------|-----------|------------|------|-------|------|-----|------|-------|--------|--------|----------|
| | Bi | Bii | Biii | Biv | Bv | Bvi | Bvii | Bviii | Bix | Bx | Bxi | Bxii | Bxiii | Bxiv | Bxv | Bxvi | Bxvii | Bxviii | Bxix | Bxx |
| H ₂ S production | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| Nitrate reduction | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| Haemolysis on blood agar | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| Lipase production on EYA | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| Digestion of gelatin | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| Indole production | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| Hydrolysis of aesculin | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| Fermentation of: | | | | | | | | | | | | | | | | | | | | |
| glucose | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| lactose | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| maltose | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| sucrose | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| rhamnose | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| trehalose | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| mannitol | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| Hydrolysis of dextran | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| Reference strains | NCTC 9343 | NCTC 10583 | NCTC 8560 | NCTC 9344 | NCTC 10581 | NCTC 10584 | ATCC 8503 | ATCC 8482 | ATCC 8483 | ATCC 8492 | NCTC 10582 | ... | ... | ... | ... | ... | ... | ... | NP 333 | VPI 2845 |
| conforming to the given pattern | 47 | 1 | 4 | 12 | 1 | 4 | 0 | 2 | 1 | 2 | 0 | 1 | 3 | 2 | 7 | 1 | 5 | 1 | 0 | 0 |
| No. of laboratory isolates conforming | 52 | 1 | 5 | 12 | 1 | 4 | 1 | 3 | 1 | 2 | 1 | 1 | 3 | 3 | 8 | 1 | 5 | 1 | 1 | 1 |
| Total no. of strains conforming | | | | | | | | | | | | | | | | | | | | |

* + = positive result; - = negative result; ± = weak reaction.

TABLE 1B
Results of biochemical tests with black-pigmented Bacteroides organisms (M-strains)

| Test | Pattern of results* | | | | | | | | | | | |
|---|---------------------|----------|------|-----------|-----------|-----|------|-------|-----------|-----|-----|-------|
| | Mi | Mii | Miii | Miv | Mv | Mvi | Mvii | Mviii | Mix | Mx | Mxi | Mxii† |
| H ₂ S production | + | + | + | + | + | + | + | + | + | + | + | + |
| Nitrate reduction | - | - | - | - | - | - | - | - | - | - | - | - |
| Haemolysis on blood agar | + | + | + | + | + | + | + | + | + | + | + | + |
| Lipase production on EYA | + | + | + | + | + | + | + | + | + | + | + | + |
| Digestion of gelatin | + | + | + | + | + | + | + | + | + | + | + | + |
| Indole production | - | - | - | - | - | - | - | - | - | - | - | - |
| Hydrolysis of aesculin | + | - | - | - | - | - | - | - | - | - | - | - |
| Fermentation of: | | | | | | | | | | | | |
| glucose | + | + | + | + | + | + | + | + | + | + | + | + |
| lactose | + | + | + | + | + | + | + | + | + | + | + | + |
| maltose | + | + | + | + | + | + | + | + | + | + | + | + |
| sucrose | + | + | + | + | + | + | + | + | + | + | + | + |
| rhamnose | - | - | - | - | - | - | - | - | - | - | - | - |
| trehalose | - | - | - | - | - | - | - | - | - | - | - | - |
| mannitol | - | - | - | - | - | - | - | - | - | - | - | - |
| Hydrolysis of dextran | + | + | - | - | - | - | - | - | - | - | - | - |
| Reference strains conforming to the given pattern | ATCC 15930 | VPI 4196 | ... | NCTC 9338 | NCTC 9336 | ... | ... | ... | NCTC 9337 | ... | ... | ... |
| No. of laboratory isolates conforming | 0 | 0 | 1 | 11 | 1 | 4 | 1 | 4 | 7 | 1 | 1 | 5 |
| Total no. of strains conforming | 1 | 1 | 1 | 12 | 2 | 4 | 1 | 4 | 8 | 1 | 1 | 5 |

* + = positive result; - = negative result.

† Five laboratory isolates that did not grow on EYA are shown in the Tables as biochemical group Mxii.

TABLE 1C
Results of biochemical tests with miscellaneous organisms of the Bacteroides-Fusobacterium group (*F-strains*)

| Test | Pattern of results* | | | | | | | | | |
|---|---------------------|--|------|------------|------------|------------|------|-------|-----------|------------|
| | Fj | Fii | Fiii | Fiv | Fv | Fvi | Fvii | Fviii | Fix | Fx |
| H ₂ S production | + | + | - | + | - | + | - | + | + | - |
| Nitrate reduction | + | - | - | - | - | - | - | - | - | + |
| Haemolysis on blood agar | + | + | + | - | - | - | - | - | - | + |
| Lipase production on EYA | - | + | + | - | - | - | - | - | - | - |
| Digestion of gelatin | + | - | - | + | - | - | - | - | - | - |
| Indole production | - | + | - | - | - | - | - | - | - | - |
| Hydrolysis of aesculin | - | - | - | - | + | + | - | - | + | - |
| Fermentation of | | | | | | | | | | |
| glucose | - | + | + | - | + | - | + | - | + | - |
| lactose | - | - | - | - | + | - | + | - | + | - |
| maltose | - | + | + | - | + | - | + | - | + | - |
| sucrose | - | + | - | - | + | - | + | - | + | - |
| rhamnose | - | - | - | - | - | - | - | - | - | - |
| trehalose | - | - | - | - | + | + | - | - | - | - |
| mannitol | - | - | - | - | - | - | - | - | - | - |
| Hydrolysis of dextran | - | - | - | - | - | - | - | - | - | - |
| Reference strains conforming to the given pattern | ... | NCTC 10575 NCTC 10576 NCTC 10577 | ... | NCTC 10562 | NCTC 10249 | NCTC 10723 | ... | ... | NCTC 7155 | NCTC 10206 |
| No. of laboratory isolates conforming | 4 | 0 | 1 | 1 | 0 | 0 | 1 | 2 | 0 | 0 |
| Total no. of strains conforming | 4 | 3 | 1 | 2 | 1 | 1 | 1 | 2 | 1 | 1 |

* + = positive result; - = negative result; ⊥ = weak reaction.

The five reference strains of *B. fragilis* ss. *fragilis* and 47 strains isolated from clinical specimens gave pattern Bi. *B. fragilis* ss. *vulgatus* NCTC 10583 and 4 laboratory isolates gave pattern Biii, distinguished by the ability to digest gelatin and ferment rhamnose. Pattern Biv, obtained with 12 strains from normal human faeces differed from Biii in only one respect (aesculin hydrolysis negative); *B. fragilis* ss. *vulgatus* ATCC 8482 was significantly different and gave a pattern (Bviii) similar to that obtained with *B. fragilis* ss. *distasonis* ATCC 8503 (Bvii). *B. fragilis* ss. *ovatus* ATCC 8483 (pattern Bxi) was the only strain that fermented mannitol. Nine laboratory isolates gave the same patterns (Bxiv and Bxv) as the two reference strains of *B. fragilis* ss. *thetaitaomicron* ATCC 8492 and NCTC 10582 that differed only in the ability to hydrolyse dextran. *B. oralis* NP 333 and *B. ochraceus* VPI 2845 gave individual patterns (Bxix and Bxx) that differed only in the fermentation of rhamnose. The 20 remaining laboratory isolates gave patterns that were intermediate between the patterns obtained with the reference strains.

The 'M-strains'. Twelve patterns of results (Mi-Mxii) were obtained from the biochemical tests on 41 M-strains (Table 1b). All strains produced black-pigmented colonies on blood agar surrounded by a zone of clear haemolysis and all except one strain digested gelatin. The patterns of results with most strains fell within two groups clearly differentiated by the results of carbohydrate-fermentation tests.

B. melaninogenicus NCTC 9336 and 9338 and 12 laboratory isolates—10 from human gingival crevice material and dental plaque and 2 from clinical specimens—gave similar patterns (Miv and Mv) that differed only in the production of lipase; five strains (patterns Miii and Mvi) differed only in the fermentation of lactose.

The second group were asaccharolytic. *B. melaninogenicus* NCTC 9337 and 11 laboratory isolates—10 from abdominal wounds and high vaginal swabs and one oral isolate—gave similar patterns (Mviii and Mix). Five asaccharolytic strains failed to grow on supplemented egg yolk agar and are, therefore, placed in a separate pattern (Mxii). *B. melaninogenicus* ss. *melaninogenicus* VPI 4196 and *B. melaninogenicus* (formerly *B. oralis*) ATCC 15930 gave similar patterns but differed in the production of lipase and the hydrolysis of aesculin. The remaining three strains gave individual patterns (Mvii, Mx and Mxi). In particular, the strains that gave patterns Mx and Mxi were the only *Bacteroides* strains except *B. corrodens* that reduced nitrate to nitrite.

The 'F-strains'. The results of the biochemical tests with reference strains of *Fusobacterium* spp. and a small miscellaneous group of laboratory isolates are given in Table 1c. None of these strains produced black-pigmented colonies on blood agar. The four strains of *B. corrodens* gave pattern Fi. *F. necrophorum* NCTC 10575, 10576 and 10577 gave pattern Fii; they gave variable results in fermentation tests with glucose and maltose but the results were never more than weakly positive (a fall in pH of 0.5 unit). *F. polymorphum* NCTC 10562, *F. necrogenes* NCTC 10723 and *L. buccalis* NCTC 10249 gave individual patterns of results. Two laboratory isolates were unreactive except in H₂S production (pattern Fviii), and one isolate gave positive results only weakly in some fermentation tests (pattern Fvii). *B. necrophorus* NCTC 7155 gave the pattern (Fix) of *B. fragilis* ss. *fragilis*. The pattern (Fx) obtained with *L. dentium* NCTC 10206 is included although the organism is not a true Gram negative anaerobe.

TABLE 2
Results of tolerance tests with 165 strains of Gram negative anaerobic bacilli

| Test | Pattern of results* | | | | | | | | | | | | | | Q | (R)† |
|---|---------------------|-----|-----|-----------|------------|-----|-----|-----|--------|------------|-----|------------|------------|------------|-----------|------|
| | A | B | C | D | E | F | G | H | J | K | L | M | N | P | | |
| Growth on basal medium plus: | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | ... |
| taurocholate (0.5%) | + | + | + | + | + | + | + | + | I | I | I | I | + | I | + | ... |
| deoxycholate (0.1%) | I | I | I | I | I | I | I | + | I | I | I | I | + | I | + | ... |
| taurocholate (0.5%) & deoxycholate (0.1%) | + | I | + | + | + | I | I | + | I | I | I | I | + | I | + | ... |
| Victoria blue 4R (1/80 000) | + | + | + | I | + | + | I | + | + | I | + | + | + | + | + | ... |
| ethyl violet (1/80 000) | I | I | I | I | + | + | I | I | I | I | I | + | + | + | + | ... |
| gentian violet (1/100 000) | I | I | I | I | I | I | I | I | I | I | I | + | + | + | + | ... |
| brilliant green (1/80 000) | I | I | + | I | I | I | I | I | I | I | + | + | I | I | + | ... |
| Reference strains conforming to the given pattern | NCTC 8560 | ... | ... | ATCC 8482 | NCTC 10583 | ... | ... | ... | NP 333 | NCTC 9336 | ... | NCTC 10575 | NCTC 10723 | NCTC 10249 | NCTC 7155 | ... |
| | NCTC 9343 | | | ATCC 8503 | | | | | | NCTC 9337 | | NCTC 10576 | | | | |
| | NCTC 9344 | | | | | | | | | NCTC 9338 | | NCTC 10577 | | | | |
| | NCTC 10581 | | | | | | | | | VPI 4196 | | NCTC 10562 | | | | |
| | NCTC 10582 | | | | | | | | | VPI 2845 | | | | | | |
| | NCTC 10584 | | | | | | | | | ATCC 15930 | | | | | | |
| | ATCC 8483 | | | | | | | | | NCTC 10206 | | | | | | |
| | ATCC 8492 | | | | | | | | | | | | | | | |
| No. of laboratory isolates conforming | 48 | 15 | 2 | 0 | 15 | 1 | 1 | 1 | 7 | 38 | 6 | 2 | 0 | 0 | 0 | 3 |
| Total no. of strains conforming | 56 | 15 | 2 | 2 | 16 | 1 | 1 | 1 | 8 | 45 | 6 | 6 | 1 | 1 | 1 | 3 |

* + = growth; I = inhibition of growth.

† Three laboratory isolates of *B. melaninogenicus* that did not grow on tolerance test basal medium are shown in the tables as tolerance group R.

Results of tolerance tests

Sixteen patterns (A-R) of results were obtained with the 165 test strains in tolerance tests with bile salts and dyes (Table 2). *B. fragilis* ss. *fragilis* NCTC 8560, 9343, 9344, 10581 and 10584, *B. fragilis* ss. *thetaiotaomicron* NCTC 10582 and ATCC 8492, *B. fragilis* ss. *ovatus* ATCC 8483, and 48 laboratory isolates gave pattern A. Fifteen laboratory isolates gave a similar pattern (B) but inhibition of growth by deoxycholate was not prevented by the presence of taurocholate. *B. fragilis* ss. *vulgatus* NCTC 10583 and 15 laboratory isolates grew in the presence of Victoria blue 4R and ethyl violet (pattern E). *B. fragilis* ss. *vulgatus* ATCC 8482 was inhibited by these dyes and gave the same pattern (D) as *B. fragilis* ss. *distasonis* ATCC 8503.

B. melaninogenicus NCTC 9336, 9337, 9338, ATCC 15930 and VPI 4196 and 30 laboratory isolates of *B. melaninogenicus* gave pattern K; *B. ochraceus* VPI 2845, *L. dentium* NCTC 10206, 7 laboratory isolates of *B. fragilis*-like strains, and 1 F-strain gave the same pattern. *B. oralis* NP 333, 2 laboratory isolates of *B. melaninogenicus*, 2 F-strains, and 3 *B. fragilis*-like strains gave pattern J that differed from pattern K by growth in the presence of Victoria blue 4R.

The 4 *B. corrodens* strains, 1 *B. melaninogenicus* strain and 1 *B. fragilis*-like strain gave pattern L. *F. necrophorum* NCTC 10575, 10576 and 10577, *F. polymorphum* NCTC 10562, and 2 laboratory isolates gave tolerance pattern M. *F. necrogenes* NCTC 10723, *L. buccalis* NCTC 10249, and *B. necrophorus* NCTC 7155 gave unique tolerance patterns (N, P and Q). Strain NCTC 7155 was the only test strain that grew in the presence of all the dyes and bile salts used. Three *B. melaninogenicus* strains did not grow on tolerance test basal medium (pattern (R)).

Results of antibiotic disc resistance tests

The patterns of results obtained with the 165 test strains in antibiotic disc resistance tests are shown in Table 3a and b. The 105 *B. fragilis*-like strains gave 18 patterns of resistance (1-18); 61 strains gave pattern 1; they were resistant to the test concentrations of neomycin, kanamycin, penicillin, methicillin, colistin, lincomycin, bacitracin and vancomycin. Fifteen strains (patterns 12-18) were sensitive to the 1000 µg neomycin disc, 1 strain (pattern 17) was sensitive to the 1000 µg kanamycin disc, and 2 strains (patterns 4 and 17) were sensitive to vancomycin. Six strains were sensitive to penicillin and methicillin (patterns 10 and 11) and 2 strains (pattern 18) to methicillin alone. Fourteen strains were resistant to erythromycin (patterns 2, 7, 8, 9 and 12); 3 strains were resistant to rifampicin (patterns 6, 7 and 8) and 2 strains were resistant to chloramphenicol (patterns 7 and 16). Twenty-one strains were sensitive to lincomycin (patterns 4, 5, 6, 9, 10, 14, 15, 16, 17 and 18) but all except 6 strains (patterns 3 and 8), were sensitive to clindamycin. Two strains were sensitive to colistin (pattern 18) and 3 strains were sensitive to bacitracin (patterns 15 and 17).

The 41 *B. melaninogenicus* strains gave 12 patterns of resistance (19-30). Seventeen strains gave pattern 24; they were resistant to the 10 µg neomycin disc, 30 µg and 1000 µg kanamycin discs, and vancomycin, and sensitive to the other antibiotics. Three strains were resistant to penicillin and methicillin (patterns 23 and 30), 3 to chloramphenicol (patterns 21 and 29), and 1 strain (pattern 30) was resistant to lincomycin and clindamycin. All except 5 strains (patterns 19-22) were sensitive to the 1000 µg neomycin disc; 15 strains were sensitive to vancomycin (patterns 20-22,

TABLE 3A
Results of antibiotic disc resistance tests with the test strains of Gram negative anaerobic bacilli: patterns 1-20

| Antibiotic (content per disc) | Patterns of results* | | | | | | | | | | | | | | | | | | | |
|---|----------------------|--------------|---------------|---------------|-----|--------------|--------------|-----|-----|-----|-----|--------------|-----|-----|-----|--------------|-----|-----|-----|-----|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 |
| Neomycin (1000 µg) | R | R | R | R | R | R | R | R | R | R | R | S | S | S | S | S | S | S | R | R |
| Neomycin (10 µg) | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R |
| Kanamycin (1000 µg) | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R |
| Kanamycin (30 µg) | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R |
| Penicillin (1.5 units) | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R |
| Methicillin (10 µg) | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R |
| Erythromycin (60 µg) | S | R | S | S | S | S | R | R | R | S | S | R | S | S | S | S | S | S | S | S |
| Colistin (10 µg) | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R |
| Rifampicin (15 µg) | S | S | S | S | S | S | R | R | S | S | S | S | S | S | S | S | S | S | S | S |
| Lincomycin (2 µg) | R | R | R | R | R | S | R | R | S | S | R | R | R | S | S | S | S | S | S | S |
| Clindamycin (2 µg) | S | S | R | S | S | S | S | R | S | S | S | S | S | S | S | S | S | S | S | S |
| Bacitracin (0.1 unit) | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R |
| Vancomycin (15 µg) | R | R | R | S | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R |
| Chloramphenicol (10 µg) | S | S | S | S | S | S | R | S | S | S | S | S | S | S | S | R | S | S | S | S |
| Reference strains conforming to the pattern | NCTC 9343 | NCTC 8560 | NCTC 10582 | NCTC 10581 | ... | ATCC 8492 | ATCC 8483 | ... | ... | ... | ... | ATCC 8482 | ... | ... | ... | ATCC 8503 | ... | ... | ... | ... |
| | NCTC 9344 | | | | | | | | | | | | | | | | | | | |
| | NCTC 10583 | | | | | | | | | | | | | | | | | | | |
| | NCTC 10584 | | | | | | | | | | | | | | | | | | | |
| No. of laboratory isolates conforming | 57 | 7 | 4 | 0 | 5 | 0 | 0 | 1 | 1 | 5 | 1 | 3 | 3 | 2 | 2 | 0 | 1 | 2 | 2 | 1 |
| Total no. of strains conforming | 61 | 8 | 5 | 1 | 5 | 1 | 1 | 1 | 1 | 5 | 1 | 3 | 4 | 2 | 2 | 1 | 1 | 2 | 2 | 1 |

* S = sensitive; R = resistant

TABLE 3B
Results of antibiotic disc resistance tests: patterns 21-41

| Antibiotic (content per disc) | Patterns of results* | | | | | | | | | | | | | | | | | | | | |
|---|----------------------|-----|-----|-----------|-----|-----------|------------|----------|-----|-----|----------|-----|-----|------------|-----|------------|------------|-----------|-----|-----|------------|
| | 21 | 22 | 23 | 24 | 25 | 26 | 27 | 28 | 29 | 30 | 31 | 32 | 33 | 34 | 35 | 36 | 37 | 38 | 39 | 40 | 41 |
| Neomycin (1000 µg) | R | R | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S |
| Neomycin (10 µg) | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R |
| Kanamycin (1000 µg) | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R |
| Kanamycin (30 µg) | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R |
| Penicillin (1.5 units) | S | S | R | S | S | S | S | S | S | R | S | R | R | R | S | S | S | S | S | S | S |
| Methicillin (10 µg) | S | S | R | S | S | S | S | S | S | R | S | S | R | S | S | S | S | S | S | S | S |
| Erythromycin (60 µg) | S | S | S | S | S | S | S | S | S | S | S | S | S | S | R | S | S | R | R | S | S |
| Colistin (10 µg) | S | R | S | S | S | R | R | S | S | S | R | R | S | S | S | S | S | R | S | S | S |
| Rifampicin (15 µg) | S | S | S | S | S | S | S | S | S | S | S | S | S | R | S | S | S | R | R | S | R |
| Lincomycin (2 µg) | S | S | S | S | S | S | S | S | S | R | S | S | S | S | S | S | S | R | R | S | S |
| Clindamycin (2 µg) | S | S | S | S | S | S | S | S | S | R | R | S | S | S | S | S | S | R | R | S | S |
| Bacitracin (0.1 unit) | S | R | S | S | S | S | R | R | S | R | R | R | R | R | R | S | R | R | R | R | S |
| Vancomycin (15 µg) | S | S | R | R | S | S | S | R | R | S | S | R | S | R | R | S | R | R | S | S | S |
| Chloramphenicol (10 µg) | R | S | S | S | S | S | S | S | R | S | S | S | S | S | S | S | S | S | S | S | S |
| Reference strains conforming to the pattern | ... | ... | ... | NCTC 9336 | ... | NCTC 9337 | ATCC 15930 | VPI 4196 | ... | ... | VPI 2845 | ... | ... | NCTC 10375 | ... | NCTC 10249 | NCTC 10562 | NCTC 7155 | ... | ... | NCTC 10206 |
| | | | | NCTC 9338 | | | | NP 333 | | | | | | NCTC 10376 | | | | | | | |
| | | | | | | | | | | | | | | NCTC 10377 | | | | | | | |
| | | | | | | | | | | | | | | NCTC 10378 | | | | | | | |
| No. of laboratory isolates conforming | 1 | 1 | 2 | 15 | 3 | 4 | 2 | 2 | 2 | 1 | 0 | 1 | 1 | 0 | 1 | 1 | 0 | 0 | 1 | 4 | 0 |
| Total no. of strains conforming | 1 | 1 | 2 | 17 | 3 | 5 | 3 | 4 | 2 | 1 | 1 | 1 | 1 | 4 | 1 | 2 | 1 | 1 | 1 | 4 | 1 |

* S = sensitive; R = resistant

25-27 and 30), but only 3 strains were sensitive to the 1000 µg kanamycin disc (patterns 29 and 30). Twelve strains were resistant to colistin (patterns 19, 20, 22, 26 and 27) and 8 strains were resistant to bacitracin (patterns 22, 27, 28 and 30). *B. oralis* NP 333 gave the same pattern (28) as 3 *B. melaninogenicus* strains. *B. ochraceus* VPI 2845, however, was sensitive to the 1000 µg kanamycin disc and vancomycin, and resistant to colistin, clindamycin and bacitracin (pattern 24).

The 17 F-strains gave 10 patterns of resistance (32-41). *L. dentium* NCTC 10206 was sensitive to all agents except colistin (pattern 41). All 4 strains of *B. corrodens* gave pattern 40; they were sensitive to all agents except rifampicin and bacitracin. *F. necrophorum* NCTC 10575, 10576 and 10577, and *F. necrogenes* NCTC 10723 (pattern 34) and *B. necrophorus* NCTC 7155 (pattern 38) were also resistant to rifampicin. The remaining F-strains gave varied patterns.

Comparison of results of biochemical tests with results of tolerance tests

The patterns of tolerance obtained with the 107 B-strains that gave 20 patterns of biochemical results are shown in Table 4a. Forty-nine of the 52 strains that gave the biochemical pattern Bi (*B. fragilis* ss. *fragilis*) and 19 of the 22 strains that gave patterns Bviii-Bxv and contained all *B. fragilis* ss. *ovatus* and *B. fragilis* ss. *thetaiotaomicron* strains gave tolerance patterns A and B. The 15 strains in tolerance pattern B did not grow in the presence of the deoxycholate and taurocholate mixture.

The 17 strains of *B. fragilis* ss. *vulgatus* in biochemical groups Biii and Biv gave tolerance patterns E and F, distinguished by growth in the presence of ethyl violet. Eleven strains that gave biochemical patterns conforming with the *B. fragilis*-like group (patterns Bi-Bxviii) gave tolerance patterns J, K and L that were the same as patterns obtained with *B. melaninogenicus* strains. *B. oralis* NP 333, *B. ochraceus* VPI 2845 (biochemical patterns Bxix and Bxx), *L. dentium* NCTC 10206 and three F-strains also gave tolerance patterns J and K.

Thirty-five of the 41 strains of *B. melaninogenicus* (biochemical patterns Mi-Mxii) gave tolerance pattern K (Table 4b). Three strains grew in the presence of Victoria blue 4R (tolerance patterns J and L) and the remaining 3 strains failed to grow on the tolerance test basal medium.

The patterns of tolerance obtained with 17 F-strains are shown in Table 4c. The 4 strains of *B. corrodens* that gave biochemical pattern Fi gave tolerance pattern L. *F. necrophorum* NCTC 10575, 10576 and 10577 (biochemical pattern Fii), *F. polymorphum* NCTC 10562 (biochemical pattern Fiv) and two laboratory isolates (biochemical patterns Fiv and Fviii) gave tolerance pattern M.

Comparison of results of biochemical tests with results of antibiotic disc resistance tests

The patterns of antibiotic resistance and biochemical results are compared in Table 5a, b and c. Sixty-one of the 105 *B. fragilis*-like strains (biochemical patterns Bi-Bxviii) gave resistance pattern 1 and a further 8 strains differed only in resistance to erythromycin (pattern 2). B-strains with the antibiotic resistance patterns 3-18 were distributed amongst biochemical groups Bi-Bxviii and the recognized subspecies of *B. fragilis* were not distinguishable by their antibiotic resistance patterns.

TABLE 4A
The relationship of biochemical patterns to tolerance patterns of *B*-strains

| Biochemical pattern* | Number of strains with the stated tolerance pattern† | | | | | | | | | | | | | | | | Key to position of reference strain(s) (indicated by superscript) | |
|----------------------|--|---|---|---|----------------|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | A | B | C | D | E | F | G | H | I | J | K | L | M | N | P | Q | | (R) |
| Bi | 41 ^a | 8 | 2 | | | | | | 1 | | | | | | | | | a <i>B. fragilis</i> ss. <i>fragilis</i> NCTC 9343, 9344, 10581, 10584 and 8560 |
| Bii | | | | | | | | | | | 1 | | | | | | | b <i>B. fragilis</i> ss. <i>vulgatus</i> NCTC 10583 |
| Biii | | | | | 4 ^b | 1 | | | | | | | | | | | | |
| Biv | | | | | 12 | | | | | | | | | | | | | |
| Bv | | | | | | | | | | | 1 | | | | | | | |
| Bvi | | | | | | | | | | 1 | 2 | 1 | | | | | | |
| Bvii | | | | | | | | | | | | | | | | | | |
| Bviii | | | | | | | | | | | | | | | | | | c <i>B. fragilis</i> ss. <i>distasonis</i> ATCC 8503 |
| Bix | 2 | | | | | | | | | | | | | | | | | d <i>B. fragilis</i> ss. <i>vulgatus</i> ATCC 8482 |
| Bx | | | | | | | | | | | | | | | | | | |
| Bxi | | | | | | | | | | | | | | | | | | |
| Bxii | | | | | | | | | | | | | | | | | | e <i>B. fragilis</i> ss. <i>ovatus</i> ATCC 8483 |
| Bxiii | | | | | | | | | | | | | | | | | | |
| Bxiv | | | | | | | | | | | | | | | | | | |
| Bxv | | | | | | | | | | | | | | | | | | |
| Bxvi | | | | | | | | | | | | | | | | | | |
| Bxvii | | | | | | | | | | | | | | | | | | f <i>B. fragilis</i> ss. <i>thetaiotaomicon</i> ATCC 8492 |
| Bxviii | | | | | | | | | | | | | | | | | | g <i>B. fragilis</i> ss. <i>thetaiotaomicon</i> NCTC 10582 |
| Bxix | | | | | | | | | | | | | | | | | | |
| Bxx | | | | | | | | | | | | | | | | | | h <i>B. oralis</i> NP 333 |
| | | | | | | | | | | | | | | | | | | j <i>B. ochraceus</i> VPI 2845 |

* see Table 1a; † see Table 2.

TABLE 4B
The relationship of biochemical patterns to tolerance patterns of *M*-strains

| Biochemical pattern* | Number of strains with the stated tolerance pattern† | | | | | | | | | | | Key to position of reference strain(s) (indicated by superscript) |
|----------------------|--|---|---|---|---|---|---|---|---|---|-----------------|---|
| | A | B | C | D | E | F | G | H | I | J | K | (R) |
| Mi | | | | | | | | | | | 1 ^k | k <i>B. melaninogenicus</i> ATCC 15930 |
| Mii | | | | | | | | | | | 1 ^l | l <i>B. melaninogenicus</i> VPI 4196 |
| Miii | | | | | | | | | | | 1 | |
| Miv | | | | | | | | | | | 12 ^m | m <i>B. melaninogenicus</i> NCTC 9338 |
| Mv | | | | | | | | | | 1 | 2 ⁿ | n <i>B. melaninogenicus</i> NCTC 9336 |
| Mvi | | | | | | | | | | | 3 | |
| Mvii | | | | | | | | | | | 1 | |
| Mviii | | | | | | | | | | | 4 | |
| Mix | | | | | | | | | | 1 | 7 ^p | p <i>B. melaninogenicus</i> NCTC 9337 |
| Mx | | | | | | | | | | | 1 | |
| Mxi | | | | | | | | | | | 1 | |
| Mxii | | | | | | | | | | | 2 | (3) |

* see Table 1b; † see Table 2.

TABLE 4C
The relationship of biochemical patterns to tolerance patterns of *F*-strains

| Biochemical pattern* | Number of strains with the stated tolerance pattern† | | | | | | | | | | | Key to position of reference strain(s) (indicated by superscript) |
|----------------------|--|---|---|---|---|---|---|---|---|---|----------------|---|
| | A | B | C | D | E | F | G | H | I | J | K | (R) |
| Fi | | | | | | | | | | | 4 | q <i>F. necrophorum</i> NCTC 10575, 10576 and 10577 |
| Fii | | | | | | | | | | | 3 ^a | |
| Fiii | | | | | | | | | | | 1 | |
| Fiv | | | | | | | | | | | 2 ^r | r <i>F. polymorphum</i> NCTC 10562 |
| Fv | | | | | | | | | | | 1 ^s | s <i>L. buccalis</i> NCTC 10249 |
| Fvi | | | | | | | | | | | 1 ^t | t <i>F. necrogenes</i> NCTC 10723 |
| Fvii | | | | | | | | | | 1 | | |
| Fviii | | | | | | | | | | 1 | | |
| Fix | | | | | | | | | | | 1 ^u | u <i>B. necrophorus</i> NCTC 7155 |
| Fx | | | | | | | | | | | 1 ^v | v <i>L. dentium</i> NCTC 10206 |

* see Table 1c; † see Table 2.

TABLE 5A
The relationship of biochemical patterns to antibiotic resistance patterns of *B*-strains

| Bio-chemical pattern* | Number of strains with the stated pattern of antibiotic resistance† | | | | | | | | | | | | | | | | | | | | Key to position of reference strain(s) (indicated by superscript) |
|-----------------------|---|----------------|----------------|---|---|----------------|---|---|---|----|----|----|----|----|----|----|----|----|----|----|--|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 28 | 31 | 41 |
| Bi | 36 ^a | 8 ^b | 1 ^c | 2 | 1 | 1 | 1 | 1 | 1 | 3 | | | | | | | | | | | a <i>B. fragilis</i> ss. <i>fragilis</i> NCTC 9343, 9344 and 10584 |
| Bii | | | | | | | | | | | | | | | | | | | | | b <i>B. fragilis</i> ss. <i>fragilis</i> NCTC 8560 |
| Biii | 2 ^d | | | 2 | | | | | | 1 | | | | | | | | | | | c <i>B. fragilis</i> ss. <i>fragilis</i> NCTC 10581 |
| Biv | 11 | | | 1 | | | | | | | | | | | | | | | | | d <i>B. fragilis</i> ss. <i>vulgatus</i> NCTC 10583 |
| Bv | | | | | | | | | | | | | | | | | | | | | |
| Bvi | | | | | | | | | | | | | | | | | | | | | |
| Bvii | | | | | | | | | | | | | | | | | | | | | |
| Bviii | 2 | | | | | | | | | | | | | | | | | | | | e <i>B. fragilis</i> ss. <i>distasonis</i> ATCC 8503 |
| Bix | 1 | | | | | | | | | | | | | | | | | | | | f <i>B. fragilis</i> ss. <i>vulgatus</i> ATCC 8482 |
| Bx | | | | | | | | | | 2 | | | | | | | | | | | |
| Bxi | | | | | | | | | | | | | | | | | | | | | |
| Bxii | | | | | | | | | | | | | | | | | | | | | |
| Bxiii | | | 1 | | | | | | | | | | | | | | | | | | g <i>B. fragilis</i> ss. <i>ovatus</i> ATCC 8483 |
| Bxiv | 1 | | 1 | | | | | | | | | | | | | | | | | | |
| Bxv | 4 | | 3 ^j | | | 1 ^h | | | | | | | | | | | | | | | h <i>B. fragilis</i> ss. <i>thetaiotaomicron</i> ATCC 8492 |
| Bxvi | 1 | | | | | | | | | | | | | | | | | | | | j <i>B. fragilis</i> ss. <i>thetaiotaomicron</i> NCTC 10582 |
| Bxvii | 3 | | | | | | | | | | | | | | | | | | | | |
| Bxviii | | | | | | | | | | | | | | | | | | | | | |
| Bxix | | | | | | | | | | | | | | | | | | | | | k <i>B. oralis</i> NP 333 |
| Bxx | | | | | | | | | | | | | | | | | | | | | l <i>B. ochraceus</i> VPI 2845 |

* See Table 1a; † See Table 3.

TABLE 5B
The relationship of biochemical patterns to antibiotic resistance patterns of *M*-strains

| Bio-chemical pattern* | Number of strains with the stated pattern of antibiotic resistance† | | | | | | | | | | | | | | | | Key to position of reference strain(s) (indicated by superscript) |
|-----------------------|---|---|---|---|---|---|---|---|---|----|----|----|----|----|----|----|--|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | |
| Mi | | | | | | | | | | | | | | | | | m <i>B. melaninogenicus</i> ATCC 15930 |
| Mii | | | | | | | | | | | | | | | | | n <i>B. melaninogenicus</i> VPI 4196 |
| Miii | | | | | | | | | | | | | | | | | |
| Miv | | | | | | | | | | | | | | | | | p <i>B. melaninogenicus</i> NCTC 9338 |
| Mv | | | | | | | | | | | | | | | | | q <i>B. melaninogenicus</i> NCTC 9336 |
| Mvi | | | | | | | | | | | | | | | | | |
| Mvii | | | | | | | | | | | | | | | | | |
| Mviii | | | | | | | | | | | | | | | | | |
| Mix | | | | | | | | | | | | | | | | | |
| Mx | | | | | | | | | | | | | | | | | r <i>B. melaninogenicus</i> NCTC 9337 |
| Mxi | | | | | | | | | | | | | | | | | |
| Mxii | | | | | | | | | | | | | | | | | |

* See Table 1b; † See Table 3.

TABLE 5C
The relationship of biochemical patterns to antibiotic resistance patterns of *F*-strains

| Biochemical patterns* | Number of strains with the stated pattern of antibiotic resistance† | | | | | | | | | | | | | | | | Key to position of reference strain(s) (indicated by superscript) |
|-----------------------|---|---|---|---|---|---|---|---|---|----|----|----|----|----|----|----|--|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | |
| Fi | | | | | | | | | | | | | | | | | s <i>F. necrophorum</i> NCTC 10575, 10576 and 10577 |
| Fii | | | | | | | | | | | | | | | | | |
| Fiii | | | | | | | | | | | | | | | | | |
| Fiv | | | | | | | | | | | | | | | | | t <i>F. polymorphum</i> NCTC 10562 |
| Fvi | | | | | | | | | | | | | | | | | u <i>L. buccalis</i> NCTC 10249 |
| Fvii | | | | | | | | | | | | | | | | | v <i>F. necrogenes</i> NCTC 10723 |
| Fviii | | | | | | | | | | | | | | | | | |
| Fix | | | | | | | | | | | | | | | | | w <i>B. necrophorus</i> NCTC 7155 |
| Fx | | | | | | | | | | | | | | | | | x <i>L. dentium</i> NCTC 10206 |

* See Table 1c; † see Table 3.

Sixteen saccharolytic strains of *B. melaninogenicus* and 1 asaccharolytic strain gave resistance pattern 24, but the distribution of the other 24 strains between 11 resistance patterns was unrelated to their biochemical patterns. All 4 strains of *B. corrodens* gave unique biochemical (Fi) and resistance (40) patterns. *F. necrophorum* NCTC 10575, 10576 and 10577 (biochemical pattern Fii) and *F. necrogenes* NCTC 10723 (biochemical pattern Fvi) gave the same resistance pattern (34) and the other F-strains gave varied resistance patterns.

There was no overlap between the antibiotic resistance patterns obtained with *B. fragilis*-like strains (biochemical patterns Bi-Bxviii), *B. melaninogenicus* strains (Mi-Mxii) and F-strains (Fi-Fx). *B. oralis* NP 333 (Bxix) and *B. ochraceus* VPI 2845 (Bxx) gave similar antibiotic resistance patterns to *B. melaninogenicus* strains.

Discussion

The classification of the Gram negative, non-sporing anaerobes is confused. Groups of workers have given different names to the same organism and the same name has been given to obviously different organisms (Moore & Holdeman, 1973; Holdeman & Moore, 1974). The identification of the sub-species of *B. fragilis* and *B. melaninogenicus* has presented particular problems. The difficulties in this field are partly attributable to the variety of methods used by different workers to classify their strains. In addition to conventional bacteriological approaches that include observations of microscopic and colony morphology and biochemical tests (Prévot, 1966; James & Robinson, 1975), other workers have considered numerical taxonomy (Barnes & Goldberg, 1968), gas-liquid chromatographic analysis of the acid end-products of glucose metabolism (Werner, 1969; Moore, 1970), the analysis of DNA homologies (Johnson, 1973), the ratio of the DNA bases guanine and cytosine (Sebald, cited by Barnes & Goldberg, 1968; Williams, *et al.*, 1974), serological studies of surface antigens (Sharpe, 1971), and tolerance tests that demonstrate group differences in (i) antibiotic resistance (Finegold, Harada & Miller, 1967), (ii) sensitivity to bile salts (Shimada, Sutter & Finegold, 1970), and (iii) the effects of dyes on growth (Suzuki, Ushijima & Ichinose, 1966). More recently, bacteriocin-like effects of possible use in characterization have been described in *Bacteroides* spp. (Collee, Watt & Dewhurst, 1974; Watt & Collee, 1974). It is difficult to correlate the results of these different approaches and to establish the taxonomic level at which each operates.

In the present study, conventional bacteriological tests were selected and adapted to meet the special requirements of these anaerobes. The details of the procedures given in the methods section and of the controls included in the tests are important and take account of problems of growth of strains and reproducibility of results. Findings with a few exacting strains among the 165 tested indicate that we have not solved all of the problems, but the results in general are encouraging.

The test strains fell readily into 3 main groups on the basis of our series of biochemical, tolerance and antibiotic-resistance studies: (i) non-pigmented *B. fragilis*-like strains; (ii) pigmented *B. melaninogenicus* strains; and (iii) *Fusobacterium*-like strains. The biochemical tests gave the most useful discrimination of strains within the groups. In the *B. fragilis*-like group (B-strains) there was clear separation of the reference strains representing 5 recognized sub-species of *B. fragilis*. The patterns obtained with the remaining B-strains showed clusters of strains related to the reference strains

in a spectrum that included intermediate patterns. *B. oralis* NP 333 and *B. ochraceus* VPI 2845 gave biochemical patterns that were closely related to those obtained with the *B. fragilis*-like strains.

In the *B. melaninogenicus* group (M-strains) there was clear separation of the strains into two clusters: (i) saccharolytic, and (ii) asaccharolytic; there were minor differences between strains within these clusters. Three sub-species of *B. melaninogenicus* have been described by Holdeman & Moore (1974) as *B. melaninogenicus* ss. *melaninogenicus*, ss. *intermedius*, and ss. *asaccharolyticus*. *B. melaninogenicus* NCTC 9337 and the non-fermentative laboratory isolates are clearly *B. melaninogenicus* ss. *asaccharolyticus*. *B. melaninogenicus* NCTC 9336 and 9338 conform to the published reference patterns of *B. melaninogenicus* ss. *intermedius* (Holdeman & Moore, 1973) and the remainder of our laboratory isolates of *B. melaninogenicus* were similar to these two reference strains. *B. melaninogenicus* ss. *melaninogenicus* VPI 4196, differed in a number of tests from the cluster of strains identified as *B. melaninogenicus* ss. *intermedius*. *B. melaninogenicus* (formerly *B. oralis*) ATCC 15930 was similar to but not identical with strain VPI 4196; as no laboratory isolates gave a similar pattern, it is not clear from these results whether the saccharolytic strains of *B. melaninogenicus* should be divided into two sub-species.

The F-strains were a heterogeneous collection of organisms that were clearly different from the B-strains and M-strains. There were considerable biochemical differences between reference strains of individual species within the group.

The tolerance tests largely confirmed the separation of the test strains into the three main groups. The *B. fragilis*-like strains gave a number of similar tolerance patterns; small differences were not generally related to the different sub-species, but *B. fragilis* ss. *vulgatus* NCTC 10583 and the similar laboratory isolates (but not *B. fragilis* ss. *vulgatus* ATCC 8482) gave a unique pattern. A single tolerance pattern was typical of the *B. melaninogenicus* strains but complete discrimination between B-strains and M-strains on the basis of tolerance tests was marred by a small number of B-strains; these included *B. oralis*, *B. ochraceus* and a few intermediate strains not biochemically conforming with recognized sub-species of *B. fragilis*, which gave tolerance patterns typical of *B. melaninogenicus* strains. The reference strains in the F-group were separated from the B-strains and M-strains by their tolerance patterns, although other F-strains gave similar patterns to *B. melaninogenicus* strains.

The most detailed separation between the test strains was obtained with the antibiotic disc resistance tests. There was no overlap of patterns obtained with *B. fragilis* strains, *B. melaninogenicus* strains and F-strains when the complete series of antibiotic discs was included. However, *B. oralis* NP 333 and *B. ochraceus* VPI 2845, which were classified as B-strains on the basis of biochemical tests and lack of black pigment, gave antibiotic resistance patterns that closely resembled those obtained with *B. melaninogenicus* strains. Sutter & Finegold (1971) suggested that discs containing specified concentrations of colistin, erythromycin, kanamycin, neomycin, penicillin and rifampicin could be used for preliminary identification of Gram negative anaerobic bacilli. Our results show that a significant number of strains of *B. fragilis* and *B. melaninogenicus* gave atypical results with one or more of these antibiotics. Resistance tests with neomycin 1000 µg, kanamycin 1000 µg, penicillin 1.5 units and rifampicin 15 µg were, however, useful for group discrimination in a combined approach with tolerance tests and biochemical tests. The separation of *B. fragilis* strains and *B. melan-*

inogenicus strains on the basis of antibiotic resistance patterns did not correlate with the biochemical separation of *B. fragilis* into sub-species and *B. melaninogenicus* into saccharolytic and asaccharolytic groups.

It is important that the antibiotic disc resistance tests used for identification of strains should not be confused with the sensitivity tests used to determine the susceptibility of an organism to an antibiotic for clinical use. The results of our resistance tests are not a guide to therapeutic efficacy. The method that we used was modified from that of Sutter & Finegold (1971) and the tests were done under conditions that gave good growth of the test strains. The anaerobic atmosphere accordingly contained 10% CO₂ (Watt, 1973) although its presence is known to reduce the zones of inhibition around discs containing pH sensitive antibiotics such as erythromycin and lincomycin (Ingham *et al.*, 1970). We take account of the known effect of ingredients in culture media on acid production during growth in addition to the contributory effect of CO₂ in the anaerobic atmosphere (Watt & Brown, 1975); this is of particular importance in the clinical application of sensitivity test results although the patterns obtained in resistance tests under defined conditions may still be of taxonomic value. The initial control strain for our disc resistance tests was the Oxford staphylococcus, but this was incubated anaerobically and the value of such a control continues to be debated; we are convinced of the need for a standard anaerobic organism analogous to the Oxford staphylococcus for antibiotic sensitivity testing of anaerobic organisms. Our subsequent use of *B. fragilis* NCTC 9343 as the control strain was an attempt to avoid this problem. We were unable to maintain a standard incubation period for all test strains because of the wide variation in the rates of growth of different strains. Results were therefore read as soon as growth was readily visible.

In this series of biochemical, tolerance and antibiotic resistance tests, the reference strains gave consistent results that correlated well with other published work, except that the type-strain of *B. fragilis* ss. *vulgatus*, ATCC 8482 (Holdeman & Moore, 1974), was significantly different from *B. fragilis* ss. *vulgatus* NCTC 10583 and its related cluster of laboratory isolates. Strain 8482 appeared to be more closely related to *B. fragilis* ss. *distasonis* ATCC 8503.

Dye tolerance tests (Suzuki *et al.*, 1966), studies of growth in the presence of bile salts (Shimada *et al.*, 1970) and antibiotic disc resistance tests (Finegold *et al.*, 1967) have been used for preliminary identification of Gram negative anaerobic bacilli. If these approaches were used separately, a significant number of strains in the present series would be wrongly classified because of atypical results in individual tests. Most of these problems can be overcome by using a short combined set of tolerance tests and antibiotic disc resistance tests and a small number of biochemical tests. The occurrence of atypical results would then be clear.

The set of tests shown in Table 6 would identify the major species and the sub-species of *B. fragilis* and *B. melaninogenicus* and enable a preliminary allocation of the intermediate strains into the appropriate main group. The tolerance tests and antibiotic disc resistance tests are not used in a sequential manner but as a single combined set of tests for the provisional identification of strains. They are of confirmatory value for typical *B. melaninogenicus* strains but are important for the early detection of strains that develop pigment only after prolonged incubation. This Table is based principally upon the results that we obtained with reference strains. It has proved useful for the identification of the unknown laboratory isolates although

TABLE 0
Scheme for the identification of Gram negative anaerobic bacilli

| | Result obtained in test* | | | | | |
|--|--------------------------|---|---|---|---|----|
| Pigment production† Antibiotic disc | | | | | | |
| resistance tests: | | | | | | |
| neomycin (1000 µg) | R† | S | | | | S† |
| kanamycin (1000 µg) | R | R | | | | R |
| penicillin (1·5 units) | R | S | | | | S† |
| rifampicin (15 µg) | S | S | | | | S |
| Tolerance tests: | | | | | | |
| taurocholate | + | I | I | I | I | I |
| deoxycholate | I | I | I | I | I | I |
| Victoria blue 4R | I | I | I | I | I | I |
| ethyl violet | I | I | I | I | I | I |
| Biochemical tests: | | | | | | |
| indole production | + | + | + | + | + | + |
| digestion of gelatin | + | + | + | + | + | + |
| hydrolysis of aesculin | - | - | - | - | - | - |
| Fermentation of: | | | | | | |
| glucose | + | + | + | + | + | + |
| rhamnose | + | + | + | + | + | + |
| trehalose | + | + | + | + | + | + |
| mannitol | + | + | + | + | + | + |
| <i>B. fragilis</i> | | | | | | |
| <i>ss. thetaiotaomicron</i> | | | | | | |
| <i>ss. ovalis</i> | | | | | | |
| <i>ss. distasonis</i> | | | | | | |
| <i>ss. vulgatus</i> | | | | | | |
| <i>B. fragilis</i> | | | | | | |
| <i>B. oralis</i> | | | | | | |
| <i>B. ochraceus</i> | | | | | | |
| <i>B. corrodens</i> | | | | | | |
| <i>F. necrophorum</i> | | | | | | |
| <i>F. necrogenes</i> | | | | | | |
| <i>F. polymorphum</i> | | | | | | |
| <i>L. buccalis</i> | | | | | | |
| <i>B. melaninogenicus</i> | | | | | | |
| <i>ss. intermedius</i> | | | | | | |
| <i>ss. asaccharolyticus</i> | | | | | | |

*For details including time of recording results see *Methods*; the keys to the symbols used here are given in Tables 1, 2 and 3.

† Pigment production (black colonies) observed on lysed blood agar after anaerobic incubation for up to 7 days.

Occasional strains may give anomalous results.

§... Results of these tests are not of primary importance in the identification of these species.

Note: This gives sets of results that distinguish the species. The scheme should not be regarded as a sequential key.

Fusobacterium spp., *B. fragilis* ss. *distasonis* and ss. *ovatus*, and *B. melaninogenicus* ss. *melaninogenicus* are not adequately represented in our laboratory isolates. The validity of the patterns given for the latter species must therefore await confirmation. Holdeman & Moore (1974) stress the need for further study of the *B. fragilis*-like organisms that they consider to be a continuum of variants with clusters of strains that have been designated sub-species. The present investigation confirms that clusters of strains closely resemble reference strains of recognized sub-species with a number of intermediate strains that differ from the presently accepted sub-species.

This study has shown that it is possible to use conventional procedures to obtain good characterization of strains of Gram negative anaerobic bacilli. A combination of tolerance tests, antibiotic disc resistance tests and biochemical tests has allowed us to obtain good differentiation between species of *Bacteroides* and also to identify the major sub-species. We believe this approach will be of value in the clinical microbiological laboratory.

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49. A SCHEME FOR THE IDENTIFICATION OF CLINICALLY IMPORTANT
GRAM-NEGATIVE ANAEROBIC BACILLI BY SIMPLE BACTERIOLOGICAL TESTS

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We have subjected 220 reference strains and laboratory isolates of gram-negative non-spore-forming non-motile anaerobic bacilli to a series of simple laboratory tests that included conventional biochemical tests, tests of resistance to antibiotics, and tolerance to dyes and bile salts. These tests were poised for use with gram-negative anaerobic bacilli and allowed clear separation of strains into three main groups: *Bacteroides fragilis*, *B. melaninogenicus* and *Fusobacterium* species. Certain tests were found useful for identifying the recognised subspecies of *B. fragilis* and *B. melaninogenicus*. We have selected a short set of simple tests that have particular discriminative value and we present a scheme for the identification of unknown isolates of gram-negative anaerobic bacilli. The tests are: pigment production; antibiotic disk resistance tests with neomycin (1000 µg), kanamycin (1000 µg), penicillin (1.5 units) and rifampicin (15 µg); tolerance tests with sodium taurocholate (0.5%), sodium deoxycholate (0.1%), Victoria blue 4R (1 in 80 000), and ethyl violet (1 in 80 000); and conventional biochemical tests for the production of indole, hydrolysis of aesculin, digestion of gelatin, and fermentation of glucose, rhamnose, trehalose, and mannitol. The determination of these species and certain sub-species is of clinical significance and our procedures could be routinely applied in a conventionally equipped service laboratory.

HOLBROOK, W.P. (1976).

The isolation of Bacteroides melaninogenicus from the human mouth. Int. Ass. Dent. Res. British Division.
Abstract No.81 (J. dent. Res., in press).

81.

The isolation of Bacteroides melaninogenicus from the human mouth. W.P. Holbrook, Department of Bacteriology, University of Edinburgh Medical School.

Knowledge of the anaerobic oral bacteria has been limited by the technical difficulties involved in their reliable isolation. In this study, a solid medium modified from that of Williams, R.A.D. et al., Int. J. Syst. Bact., 25 : 298-300, 1975 was adopted for a clinical survey; selective versions contained kanamycin 75 µg/ml or kanamycin 75 µg/ml plus vancomycin 2.5 µg/ml. An enrichment broth contained kanamycin and vancomycin at these concentrations; the transport medium was VMG II (Möller, A.J.R., Odont. Tid. (Special Article), 74 : 175-232, 1966). Samples of subgingival plaque were obtained with a chisel-edged narrow wooden stick from 50 subjects. The isolation of B. melaninogenicus was compared by: (i) direct plating at the chair-side on non-selective and selective media; (ii) transport to the laboratory in VMG II and culture on non-selective and selective media; (iii) seeding of enrichment broth at the chair-side and subculture on non-selective and selective media after incubation for 24h and on non-selective medium after 48h. B. melaninogenicus ss. melaninogenicus and ss. intermedius were regularly isolated and the highest isolation rate was obtained from specimens conveyed in transport medium and cultured on kanamycin - vancomycin selective medium. Selective media gave much higher isolation rates than non-selective; the enrichment broth procedure did not improve the isolation rate. Other recognised oral Gram-negative anaerobic bacilli were isolated by these techniques.

TABLE 1.

Results obtained with the 40 test strains

| | <i>B. melaninogenicus</i> ss. <i>melaninogenicus</i> | | | | | | | | | | <i>B. oralis</i> | | | | | | | | | | <i>B. ochraceus</i> | | | |
|--|--|----------|------------|----------|----------|-----------|----------|----|----------|--------|------------------|----------|-------|--------|----|-------|---|---|---|---|---------------------|---|---|---|
| growth in air + CO ₂ * | - | | | | | | | | | | - | | | | | | | | | | + | | | |
| Pigment production* | + | | | | | | | | | | - | | | | | | | | | | - | | | |
| Antibiotic test: [†] | S | | | | | | | | | | S | | | | | | | | | | S | | | |
| Neomycin 1000µg | S | | | | | | | | | | S | | | | | | | | | | S | | | |
| Tolerance tests: [‡] | | | | | | | | | | | | | | | | | | | | | | | | |
| bile salts | I | | | | | | | | | | I | | | | | | | | | | I | | | |
| Violet blue 4R | I | | | | | | | | | | I | | | | | | | | | | I | | | |
| ethyl violet | I | | | | | | | | | | I | | | | | | | | | | I | | | |
| Indole production* | - | | | | | | | | | | - | | | | | | | | | | - | | | |
| Fermentation of: [*] | | | | | | | | | | | | | | | | | | | | | | | | |
| GLSM | + | | | | | | | | | | + | | | | | | | | | | + | | | |
| Man. T | - | | | | | | | | | | - | | | | | | | | | | - | | | |
| chamrose | - | | | | | | | | | | - | | | | | | | | | | - | | | |
| Ascorbin hydrolase* | - | | | | | | | | | | - | | | | | | | | | | - | | | |
| Dextran hydrolase* | + | | | | | | | | | | + | | | | | | | | | | + | | | |
| gelatin digestion* | - | | | | | | | | | | - | | | | | | | | | | - | | | |
| lipase production* | - | | | | | | | | | | - | | | | | | | | | | - | | | |
| Haemolysis* | + | | | | | | | | | | + | | | | | | | | | | + | | | |
| Antibiotic tests: [†] | | | | | | | | | | | | | | | | | | | | | | | | |
| metronidazole | S | | | | | | | | | | S | | | | | | | | | | S | | | |
| penicillin | S | | | | | | | | | | S | | | | | | | | | | S | | | |
| methicillin | S | | | | | | | | | | S | | | | | | | | | | S | | | |
| colistin | R | | | | | | | | | | R | | | | | | | | | | R | | | |
| G.C. analysis [§] | | | | | | | | | | | | | | | | | | | | | | | | |
| acetic | + | | | | | | | | | | + | | | | | | | | | | + | | | |
| propionic | + | | | | | | | | | | + | | | | | | | | | | + | | | |
| iso-butyric | - | | | | | | | | | | - | | | | | | | | | | - | | | |
| n-butyric | - | | | | | | | | | | - | | | | | | | | | | - | | | |
| iso-valeric | + | | | | | | | | | | + | | | | | | | | | | + | | | |
| n-valeric | - | | | | | | | | | | - | | | | | | | | | | - | | | |
| lactic | + | | | | | | | | | | + | | | | | | | | | | + | | | |
| succinic | + | | | | | | | | | | + | | | | | | | | | | + | | | |
| Referred strains conforming to the pattern | WAL 2721 | VPI 4196 | ATCC 15930 | GUI 1011 | GUI 1034 | VPI 7570A | WAL 2724 | 30 | VPI 5832 | NP 333 | JI 7CM | VPI 2845 | 1956C | 10 79B | 73 | 2467B | | | | | | | | |
| No. of laboratory isolates conforming | 1 | 1 | 2 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| Total no. of strains conforming | 1 | 1 | 2 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |

* + = positive result; - = negative result.

† S = sensitive; R = resistant.

‡ I = inhibition; + = growth.

§ see methods.

TABLE 3

Results obtained with I.C.S.B. referred strains

| Test | WAL 2721 | WAL 2724 | GUI 1011 | GUI 1034 | ATCC 15930 | VPI 30 | VPI 7570A | VPI 5832 | J1 | 7CM | 1956C | 2467B |
|---------------------------------|-------------|-------------|-------------|-------------|---------------|-----------|--------------|-------------|----|-----|-------|-------|
| Growth in air + CO ₂ | - | - | - | - | - | - | - | - | - | - | + | + |
| Pigment production | + | + | + | + | + | + | + | + | - | - | - | - |
| Tolerance tests: | | | | | | | | | | | | |
| Caucocarbonate (r) | I | I | I | I | I | I | I | I | I | + | I | I |
| deoxycarbonate (d) | I | I | I | I | I | I | I | I | I | + | I | I |
| T + D | I | I | I | I | I | I | I | I | I | + | I | I |
| Victoria blue 4R | I | + | I | I | I | I | I | I | + | + | I | + |
| ethyl violet | I | I | I | I | I | I | I | I | I | + | I | I |
| gentian violet | I | I | I | I | I | I | I | I | I | I | I | I |
| brilliant green | I | I | I | I | I | I | I | I | I | I | I | I |
| Indole production | - | - | - | - | - | - | - | - | - | - | - | - |
| Fermentation of: | | | | | | | | | | | | |
| glucose | + | + | + | + | + | + | + | + | + | + | + | + |
| lactose | + | + | + | + | + | + | + | + | + | + | + | + |
| sucrose | + | + | + | + | + | + | + | + | + | + | + | + |
| maltose | + | + | + | + | + | + | + | + | + | + | + | + |
| trehalose | - | - | - | - | - | - | - | - | - | - | - | - |
| mannitol | - | - | - | - | - | - | - | - | - | - | - | - |
| rhamnose | - | - | - | - | - | - | - | + | + | + | - | - |
| Asculin hydrolysis | - | - | + | + | + | + | + | + | + | - | - | + |
| Dextran hydrolysis | - | + | - | - | + | + | - | - | - | - | + | + |
| gelatin digestion | - | - | + | + | + | - | + | - | - | - | - | - |
| lipase production | - | - | - | - | + | + | - | - | - | - | - | - |
| Haemolysis | + | + | + | + | + | + | + | + | + | + | - | - |
| H ₂ S production | + | + | + | + | + | + | + | + | + | + | + | + |
| Oxidase production | - | - | - | - | - | - | - | - | - | - | - | - |
| Catalase production | - | - | - | - | - | - | - | - | - | - | - | - |
| Antibiotic tests: | | | | | | | | | | | | |
| neomycin 1000 µg | S | S | S | S | S | R | S | S | S | R | S | S |
| neomycin 10 µg | R | R | R | R | R | R | R | R | R | R | R | R |
| kanamycin 1000 µg | R | R | R | R | R | R | R | R | R | R | R | R |
| kanamycin 30 µg | R | R | R | R | R | R | R | R | R | R | R | R |
| penicillin 1.5 units | R | S | S | S | S | S | S | S | S | R | S | S |
| methicillin 10 µg | R | S | S | S | S | S | S | S | S | R | S | S |
| erythromycin 60 µg | S | S | S | S | S | S | S | S | S | S | S | S |
| tetracycline 10 µg | R | S | S | S | S | S | S | S | S | S | S | S |
| colistin 10 µg | R | S | S | S | R | R | S | R | R | R | R | R |
| rifampicin 15 µg | S | S | S | S | S | S | S | S | S | S | S | S |
| lincomycin 2 µg | S | S | S | S | S | S | S | S | S | S | S | S |
| clindamycin 2 µg | S | S | S | S | S | S | S | S | S | S | S | S |
| bacitracin 0.1 unit | R | R | R | R | R | R | R | R | R | R | R | R |
| vancomycin 15 µg | R | R | R | R | S | R | R | R | R | R | S | R |
| chloramphenicol 10 µg | S | S | S | S | S | S | S | S | S | S | S | S |
| metronidazole 5 µg | S | S | S | S | R | R | S | S | S | S | R | R |
| G. L. C. analysis | | | | | | | | | | | | |
| acetic | + | + | + | + | + | + | + | + | + | + | + | + |
| propionic | + | + | + | + | + | + | + | + | + | + | + | + |
| iso-butyl | + | - | - | - | - | - | - | - | - | - | - | - |
| n-butyl | - | - | - | - | - | - | - | - | - | - | - | - |
| iso-valeric | + | + | + | + | - | - | + | - | - | - | + | - |
| n-valeric | - | - | - | - | - | - | - | - | - | - | - | - |
| lactic | + | + | + | + | - | - | + | - | - | - | + | - |
| succinic | + | + | + | + | + | + | + | + | + | + | + | + |

+ pale brown after incubation for 7 days.